University of Alberta

Response of Transitional Cell Carcinoma of the Bladder to TRAIL

by

Bo Sun

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Abstract

Transitional cell carcinoma of the bladder (TCCB) is the 2nd most common urologic neoplasm and the 12th leading cause of cancer deaths. Intravesical therapies are usually required after initial endoscopic surgery, due to the high probability of recurrence and progression of TCCB. TRAIL (TNF-related apoptosis-inducing ligand) has the potential for clinical application as a novel anti-TCCB agent, since it can selectively induce apoptosis in tumor cells but not in normal cells. The work in this thesis revealed that TCCB cell lines exhibit differential response to TRAIL. The amount of Bid cleavage appears to be correlated with the TRAIL sensitivity of partially resistant TCCB cells, suggesting a crucial role of tBid-mediated mitochondrial pathway in the TRAIL-induced apoptosis. Furthermore, downregulation of Bcl-2 by antisense oligodeoxynucleotides (ASO) significantly sensitizes the TRAIL-partially resistant TCCB cells to TRAIL, which provide evidence of TRAIL-ASO combination intravesical therapy as a promising novel strategy in TCCB treatment.

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List of Abbreviations

AIF	Apoptosis inducing factor
AP	Ammonium persulfate
Apaf-1	Apoptosis protease activating factor-1
APC	Antigen presenting cell
ASO	Antisense oligodeoxynucleotides
BAK	BCG-activated killing
BCG	Bacillus Calmette-Guerin
Bcl-2	B-cell lymphoma protein 2
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
BM	Basement membrane
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CARD	Caspase-recruitment domain
Caspases	Cysteinyl aspartate proteinases
CIS	Carcinoma *in situ*
CLI	Complement cytolysis inhibitor
CRD	Cysteine-rich domain
CTL	Cytotoxic T-lymphocyte
DD	Death domain
DcR	Decoy receptor
DED	Death effector domain
DFF40	DNA fragmentation factor 40
DFF45	DNA fragmentation factor 45
DIABLO	Direct IAP-associated binding protein with low pI

DISC	Death-inducing signaling complex
DL	Death ligand
DMSO	Dimethyl sulfoxide
DR	Death receptor
DTT	Dithiothreitol
DXR	Doxorobucin
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
HRP	Horseradish peroxidase
HSP	Heat shock protein
IAP	Inhibitor of apoptotic protein
ICAD	Inhibitor of caspase-activated DNase
ICE	Interleukin-1β-converting enzyme
IFN	Interferon
IL	Interleukin
LAK	Lymphokine-activated killing
MAK	Macrophage-activated killing
MCS	Multicellular spheroids
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NK	Natural kill
ODN	Oligodeoxynucleotides

OPG	Osteoprotegerin
PAGE	Polyacrylamide gel electrophoresis
PAK	P21-activated kinase
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PMSF	Pheylmethysulfonyl fluoride
RES	Reticuloendothelial system
Rb	Retinoblastoma
RT	Room temperature
S.E.	Standard error
SDS	Sodium dodecyl sulfate
Smac	Second mitochondria-derived activator of caspases
SP-40	Secreted protein 40
tBid	Truncated Bid
TCCB	Transitional cell carcinoma of the bladder
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR-associated death domain
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Tris-HCl	Trizma hydrochloride
TUR	Transurethral resection
Tween-20	Polyoxyethylene-sorbitan monolaurate
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

1.1. Thesis Overview

Transitional cell carcinoma of the bladder (TCCB) is the 5th most common neoplasm and 12th leading cause of cancer deaths [1]. About 80% of the tumors initially present as superficial TCCB [2]. The majority of TCCB are amendable to intravesical (topical) therapy after the initial tumor resection. However only high risk TCCB is normally treated with intravesical therapy due to the high recurrence ratio and the probability of progression. Bacillus Calmette-Guerin (BCG) immunotherapy has the best response rate (70%) and is considered the treatment of choice to date [3]. Although BCG is effective in treating carcinoma *in situ* (CIS) and preventing recurrence, the non-specific cytokine response appears to only delay the inevitable relapse with significant risks or side effects from this live mycobacterium [4,5]. Therefore, better intravesical strategies are required to induce apoptosis selectively in transformed cells [6]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), one of the members in tumor necrosis factor (TNF) family, could trigger apoptosis in a broad range of cancer cells, but not in normal cells [7]. Moreover, TRAIL is believed to play a role in BCG-induced anti-tumor effects in the treatment of bladder cancer [8], which provides rational support for exploring TRAIL as a potential intravesical agent.

TRAIL, also called as Apo-2 ligand, is a type II transmembrane protein with a intracellular amino-terminal tail and extracellular carboxy-terminal portion [9,10]. The extracellular domain forms a bell-shaped structure of the receptor-binding site [11], and can be proteolytically cleaved from the cell surface to yield a vesicle-associated or a soluble, biologically active form [12]. Following the binding of TRAIL, the Death Receptor 4 (DR4/TRAIL Receptor 1) or/and Death Receptor 5 (DR5/TRAIL Receptor 2)

initiate the extrinsic apoptotic pathway by recruiting Fas-associated death domain (FADD, the adaptor) and caspase-8 to form the death-inducing signaling complex, which is usually called DISC [13]. The caspase-8 recruitment in the DISC leads to its autocatalytic activation [14]. After that, the cleaved active subunits of caspase-8 are released into the cytosol where they activate the downstream caspase-3 [15]. The activated caspase-3 subunit in turn cleaves its substrates such as DNA fragmentation factor 45 (DFF45), leading to apoptosis. The cleavage of DFF45/ICAD (inhibitor of caspase-activated DNase) causes the activation of caspase-activated DNase (DFF40/CAD) and the DNA fragmentation, which is the hallmark of apoptotic cell death [16]. TRAIL might also trigger the mitochondrial pathway through BH3 interacting domain death agonist (Bid). Bid is cleaved by the active subunit of caspase-8 after TRAIL binding to DR4/5. The truncated Bid (tBid) translocates to the mitochondrial membrane and induces the release of apoptotic factors such as cytochrome *c* [17]. Sequentially, cytochrome *c* binds to apoptosis protease activating factor-1 (Apaf-1) and then associates with caspase-9 to form an apoptosome where caspase-9 is activated. The activation of capase-9 in turn causes the cleavage of caspase-3, which then leads to endonuclease fragmentation of cellular DNA [18]. Taken together, TRAIL may drive not only the death receptor (extrinsic) pathway but also the tBid-mediated mitochondrial (intrinsic) pathway to induce apoptosis. Although TRAIL-induced apoptosis has been well researched in several malignancies, the response of TCCB cells to TRAIL has not been reported in detail. Thus, the focus of this thesis was to study TRAIL-induced apoptosis in TCCB cells and explore the therapeutic potential of intravesical application.

Study results provided in this thesis demonstrated that the TCCB cell lines had different sensitivity to TRAIL. Moreover, TRAIL-induced apoptosis in responsive TCCB cells was found to be mediated through both the death receptor pathway and the tBid-mediated mitochondrial pathway. In addition, the amount of Bid cleavage appeared to be correlated with the sensitivity to TRAIL, especially in TRAIL-partially resistant TCCB cells. Further by using antisense oligodeoxynucleotides (ASO), downregulation of anti-apoptotic protein Bcl-2 in mitochondrial pathway was found to significantly enhance the sensitivity of TRAIL-partially resistant TCCB cells to TRAIL. This translational study strongly suggests a therapeutic potential of TRAIL-ASO combination strategies in the future TCCB treatment.

1.2. Transitional Cell Carcinoma of the Bladder

1.2.1. Structure and Physiology of Bladder

The bladder is a sac-like muscular organ that sits in the pelvis and is located just behind the pelvic bone (Figure 1.1A). As part of the lower urinary tract, the bladder functions as an elastic (low pressure) storage compartment, collecting the urine produced by the kidneys prior to disposal by urination. The ureters are the tubular structures that propel urine by peristalsis from the kidneys into the bladder. Urine exits the bladder via the urethra, which is of varying complexity and subcomponents depending on gender.

Structurally, the bladder wall consists mainly of four layers: the mucosa, submucosa, detrusor muscle and perivesical soft tissue (Figure 1.1B). The **mucosa** is the innermost layer and is composed of **transitional cell epithelium**, which is the site most bladder cancers originate from. Since the transitional epithelium also lines urethra, ureters and the pelvis of kidneys, the same types of cancers seen in the bladder can arise

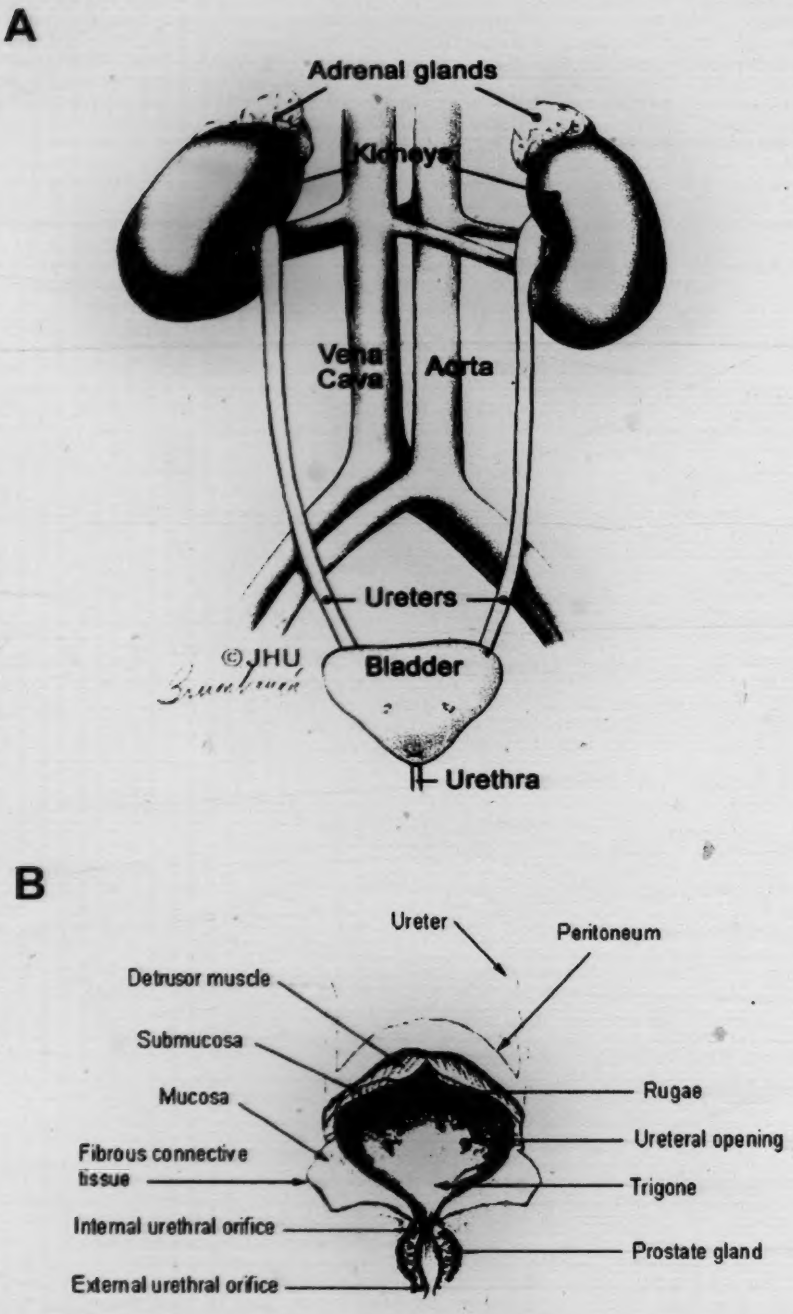


Figure 1.1: Anatomy of urinary bladder. Adapted from http://training.seer.cancer.gov/module_anatomy/images/illu_bladder.jpg

at these sites as well. The **submucosa** (also called lamina propria) lies underneath the transitional epithelium and is a layer of connective tissue with abundant blood vessels, which supply the mucosa with nutrients. The **detrusor muscle** layer consists of thick smooth muscle fibers that expand to store urine and contract to expel urine through urethra. The outermost layer of the bladder wall is the **perivesical soft tissue**, which consists of fat, fibrous tissue, as well as blood vessels. In tumorigenesis, these layers are crucial landmarks in determining tumor progression and the ultimate stage of the cancer.

1.2.2. Background knowledge of TCCB

1.2.2.1. Risk Factors of TCCB

Bladder cancer is the second most common urologic malignancy in the Western world. Over 90% of urothelial derived bladder carcinomas are transitional cell carcinomas (TCC) while squamous cell carcinomas just account for 6-8% and approximately 2% are adenocarcinomas [19]. Unlike other malignancies, such as colon cancers where the multi-step accumulation of gene mutations has been identified, the molecular mechanism that leads to bladder cancer is not entirely clear. As well, there is no evidence showing the correlation between inherited genetic mutations and bladder cancer running in family. Recently, with advances in the understanding of bladder cancer, it is believed that the accumulation of acquired mutations in both tumor suppressor genes (e.g. p53 and Rb) and proto-oncogenes (e.g. FGFR3, Ras, and HER-2/*neu*) may occur due to exposure to certain toxic chemicals in tobacco smoke or industrial pollutants that are absorbed into blood, concentrated in the urine and stored in bladder [20-25]. In addition, the certain cytotoxic chemotherapeutic drugs, such as cyclophosphamide (Cytoxan) and ifosfamide (Ifex), are also known to increase the risk of bladder cancer.

Moreover, several studies found that a 4-fold increase in the risk of bladder cancer originates from pelvic radiotherapy in women receiving the treatment for cervical cancer [26], and bladder cancer was more common in men receiving radiotherapy for prostate cancer [27]. Knowing about these factors and further investigation may help to prevent TCCB in the individuals who are potentially at risk.

1.2.2.2. TCCB Subtypes and Progression

Most cases of TCCB (around 80%) present initially as superficial disease [2]. By conventional TNM system, TCCB can be divided into several stages (Figure 1.2) [28-30]. Carcinoma *in situ* (CIS or Tis) refers to the cancers where the abnormal proliferation of tumor cells stay confined to the "superficial" mucosa and is often associated with concomitant high grade, invasive TCC in adjacent or distant urothelium. The non-invasive papillary carcinomas (Ta) are superficial and exophytic tumors, which are usually well-differentiated and do not penetrate the basement membrane (BM). With further tumor progression or when the tumor acquires the enzymatic ability to invade, early cellular invasion through the BM and lamina propria but not the muscle layer can be observed. Such tumors are described as stage T1. Sequentially, the tumor can grow into muscle (T2a/b) and perivesical soft tissue (T3a/b), spread out of the bladder (T4) and metastasize to lymph nodes (N1) or distantly (M1). Dependent on stage, multi-focally and treatment modality, more than 60% of the superficial tumors will relapse, and 15-20% will progress to invasive disease [31]. Despite the advances in standard treatments for these tumors, such as surgery, radiotherapy and chemotherapy, 50% of patients with muscle-invasive disease will ultimately die of cancer [32].

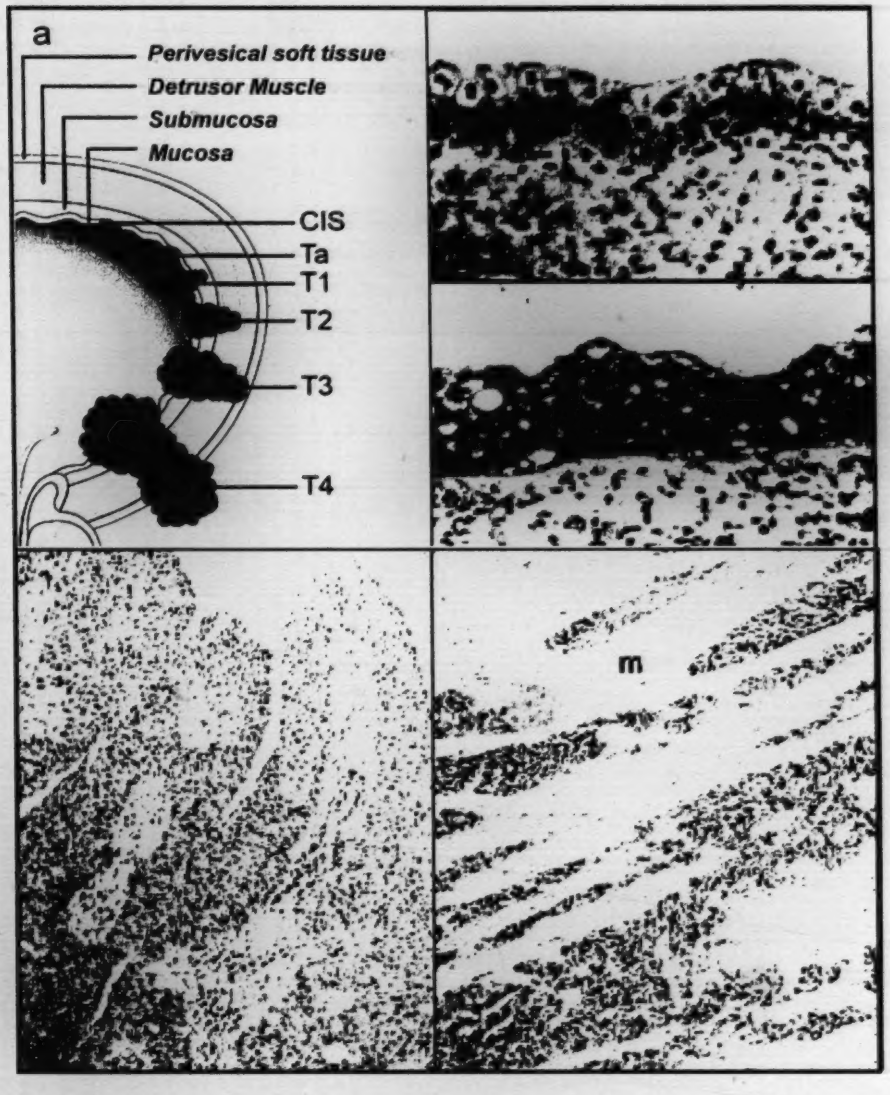


Figure 1.2: Bladder cancer stages and progression. (a) Staging of bladder cancer. Haematoxylin and eosin staining of (b) normal uroethelium, (c) carcinoma *in situ* where the tumor cells are disorganized and show marked nuclear atypia, (d) low-grade superficial papillary tumor, and (e) High-grade tumor invading muscle *(m)*. Adapted from http://www. cancerhelp.org.uk/help/default.asp?page=2703#T and reference [30].

1.2.3. Therapeutic Strategies for TCCB

The treatment of TCCB is different for superficial tumors and muscle invasive tumors. The muscle invasive tumor is usually treated with cystectomy (surgical removing the bladder), with some patients electing an attempt at bladder preservation with combination chemotherapy and radiotherapy. The superficial TCC of the bladder is usually treated with transurethral resection (endoscopic surgery). Although progression is quite rare (less than 9%) for stage Ta lesions with the transurethral resection alone, more than 60% of the cases recur [28,33]. Association with Tis carries a higher progression rate (>20%) and is difficult to visualize within the bladder. The high recurrence ratio and the probability of disease progression with superficial TCC support intravesical therapy after initial tumor resection.

1.2.3.1. Intravesical Chemotherapy

Intravesical therapies include cytotoxic chemotherapy (Thiotepa, Adriamycin Doxorubincin, Mitomycin-c, etc.) and non-specific immunotherapy (Bacillus Calmette Guerin, Keyhole Limpet Factor, interferon etc.). In addition, some of the cytotoxic agents also exert a non-specific inflammatory (immune) response. The procedures of intravesical therapies are similar no matter which chemotherapeutic agent is used. The bladder is first emptied with a catheter, and the chemotherapeutic agent is then instilled into the bladder through the same transurethral catheter. Considering the possibility of systemic absorption of the agent, avoiding trauma or injury to the urothelium is the major concern during this procedure. Theoretically, the longer the exposure time to the agents the more anti-tumor effects they will exert. However, the prolonged instillation time will

also increase cell toxicity to normal tissue and, as well, smaller molecular weight agents will be absorbed systemically. In general, the intravesical chemotherapy appears to be valuable in decreasing recurrence in the short-term after transurethral resection, but has no long-term advantage or effect on preventing disease progression [34,35]. Conversely, intravesical immunotherapy, especially the best intravesical agent to date, Bacillus Calmette Guerin (BCG), shows efficacy not only in the reduction of tumor recurrence, but also in preventing progression to muscle invasion especially for Tis and T1 disease [36-38].

1.2.3.2. Intravesical Immunotherapy

BCG, a bacterium used in vaccination against tuberculosis, is the most commonly used immune stimulant. Morales et al. first described the anti-tumor effect of BCG on the treatment of bladder cancer in 1976 [39], but even now, the exact mechanism has not been elucidated completely. It is believed that during BCG intravesical instillation, the live BCG organisms initiate an immune response on binding to the urothelium, which is facilitated through the physicochemical interaction and specific fibronectin-mediated adherence of BCG [40,41]. The BCG is then internalized by the urothelial tumor cells [42], leaving lipoproteins and glycoproteins as antigens on the cell surface to mediate the immune response [43,44]. In the immune response to BCG, the presentation of bacterial antigens by antigen-presenting cells (APC) to T-helper cells is the essential interaction, and the production of cytokines and chemokines, secreted partially by BCG-internalized tumor cells, contributes to the local activation of the immune system [8,45]. As a result, the activated leucocytes and mononuclear cells associates with particular cytokines (IFN-γ, IL-2, IL-12, and TNF-β) that promote delayed-type hypersensitivity response,

cytotoxic cell response, and macrophage activation or cellular immune inflammatory reaction, bring in the Th1 response. Several *in vitro* studies have also shown the evidence that the NK (natural killer) cells, BAK (BCG-activated killer) cells, MAK (macrophage-activated killer) cells, LAK (lymphokine-activated killer) cells and cytotoxic T lymphocytes are involved in the killing of the tumor cells [46]. Significantly, the upregulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was detected in those patients who responded to BCG, suggestive of a crucial role of TRAIL in the BCG-induced antitumor effects [8]. However depending on bacterial and host components, an upregulation of the Th2 immunoresponse, coupled with IL family cytokines, may arise to a certain extent that unfavorably affects the function of the Th1 response [47,48].

Although BCG has an excellent effect on preventing tumor from recurrence and progression in high risk superficial bladder cancer patients, the toxicity of BCG must be taken into consideration. With the BCG-induced cystitis – a local side effect, approximately 90% of the BCG-treated patients have urinary frequency, dysuria, and occasionally superpubic pain [49]. The systemic side effects of BCG, such as fever, chills, sweats, arthralgias, myalgias, and malaise can occur occasionally, whereas serious systemic complications are rare [49,50]. Further, Herr has reported that up to 39% of the patients with high-grade Ta cancers progress to muscle invasion even after transurethral resection and intravesical BCG therapy [51]. Considering conventional intravesical therapies have significant side effects and mostly delay (not cure) these early cancers, new treatment strategies are required. A treatment strategy now being explored is the

induction of selective apoptosis, in which cell content does not leak out and consequently does not lead to inflammation [6].

1.3. Apoptosis and TRAIL

1.3.1. Apoptosis: Programmed Cell Death

Apoptosis is a highly orchestrated process for noninflammatory, programmed cell death [52]. Morphologically, apoptosis is generally defined by a series of sequential events: chromatin condensation, membrane blebbing, cell shrinkage, and DNA degradation [53]. The resultant apoptotic bodies (bubbled fragments) are then quickly removed by phagocytes or adjacent cells without causing inflammation (Figure 1.3). Thus, it is different from necrosis which is uncontrolled cell death and is characterized by cell swelling, organelle/cell membrane lysis, and release of intracellular contents, leading to an inflammatory response to the surrounding cells (Figure 1.3). In addition, as compared with necrosis that is always triggered by pathological damage, apoptosis is an essential physiological activity that is evolutionarily conserved to control cell numbers, shape organs, and remove pathogen-invaded cells or auto-aggressive immune cells. As a result, the dysregulation of apoptosis may lead to development defects, neuron/autoimmune diseases, as well as cancers.

1.3.1.1. Caspases: Executioners of Apoptosis

With intense studies in the past decade, it is now clear that the central executioners of apoptosis in eukaryotic cells are a family of cysteinyl aspartate proteinases known as caspases [54-56]. The first recognized member of this family is caspase-1, which was originally named as interleukin-1β-converting enzyme (ICE) and

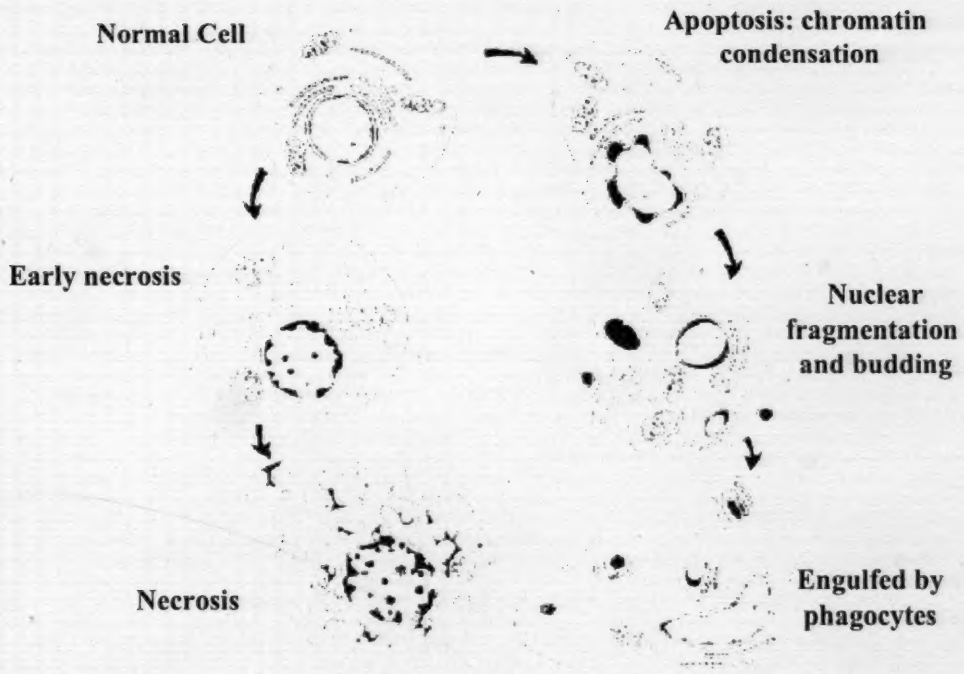


Figure 1.3: Differences between necrosis and apoptosis. Necrosis always occurs under pathological conditions, characterized by influx of intracellular ions and water, rupture of cells, and release of cytoplasmic content. Therefore, necrosis is frequently associated with an intense inflammatory response and tissue damage. In contrast, apoptosis usually occurs under normal physiological conditions, characterized by a series of morphological and biochemical changes (e.g. chromatin condensation, nuclear fragmentation, apoptotic body formation, etc.) where the cells are active participants in their programmed cell death. Adapted from Kerr *et al.,* Programmed Cell Death.

required for the activation of IL-1β [57]. Since then, a total fourteen caspases have been identified, all of which contain a conserved pentapeptide active site 'QACXG' (X can be R, Q or D) and cleave their substrates following specific aspartic residues (Table 1.1) [58,59]. Based on their biological activities, the family of caspases can be subdivided into three subfamilies: initiators of apoptosis (caspase-2, -8, -9, -10), effectors of apoptosis (caspase-3, -6, -7), as well as mediators of inflammation (caspase-1, -4, -5, -11, -12, -13, -14) (Table 1.1) [54,59,60]. In this thesis study, we focus on the caspases that function in apoptosis.

Caspases are originally synthesized as inactive proenzymes (also called zymogens or procaspases), comprising an N-terminal prodomain, a large subunit and a small subunit (Figure 1.4) [58]. Upon receiving an apoptotic signal, the procaspases are usually activated by proteolytic cleavage at the Asp-X sites between the prodomain and the large subunit domain, as well as between the large and the small domains (Figure 1.4). Although the cleavage of the proenzyme is not always mandatory for caspase activation, all the activated caspases can be detected as cleaved forms in apoptotic cells [61,62] and the matured caspase is believed to be a heterotetramer containing two heterodimers and two catalytic sites [58]. Functionally, the different prodomains of procaspases are also crucial in determining their individual roles in the apoptotic caspase-cascade system. As compared with the 22 amino acid prodomains of effector caspases (e.g. caspase-3, -6), the initiator caspases have a relatively longer prodomain (~200 amino acids) that contains specific protein-protein interaction domains [60-62]. These prodomains of initiator caspases contain either a caspase-recruitment domain (CARD, as in caspase-2 and -9), or a pair of death effector domains (DEDs, as in caspase-8 and -10) [63], which can recruit

Table 1.1: Members of the caspase family

Caspase	Other names	Active site	Cleavage site(s)
Apoptosis activators			
Caspase-8	MACH, FLICE, Mch5	QACQG	VETD↓S; LEMD↓L
Caspase-9	ICE-LAP6, Mch6	QACGG	DQLD↓A
Caspase-10	Mch4	QACQG	SQTD↓V; IEAD↓A
Caspase-2	Nedd2, ICH-1	QACRG	DQQD↓G; EESD↓A
Apoptosis executioners			
Caspase-3	CPP32, Yama, Apopain	QACRG	IETD↓S
Caspase-6	Mch2	QACRG	DVVD↓N; TEVD↓A
Caspase-7	Mch3, ICE-LAP3, CMH-1	QACRG	IQAD↓S
Inflammatory mediators			
Caspase-1	ICE	QACRG	WFKD↓S; FEDD↓A
Caspase-4	ICE_{rel}II, TX, ICH-2	QACRG	WRVD↓S; LEED↓A
Caspase-5	ICE_{rel}III, TY	QACRG	WRVD↓S; LEAD↓S
Caspase-11	ICH-3, ICE_B	QACRG	(I/L/V/P)EHD↓
Caspase-12	ICE_C	QACRG	N/A
Caspase-13	ERICE	QACRG	N/A
Caspase-14	Mini-ICE	QACRG	N/A

Adapted from Fan *et al.*, Acta Biochimica et Biophysica Sinica 2005, Kang *et al.*, J. Cell Biology 2000, and ref. [59]

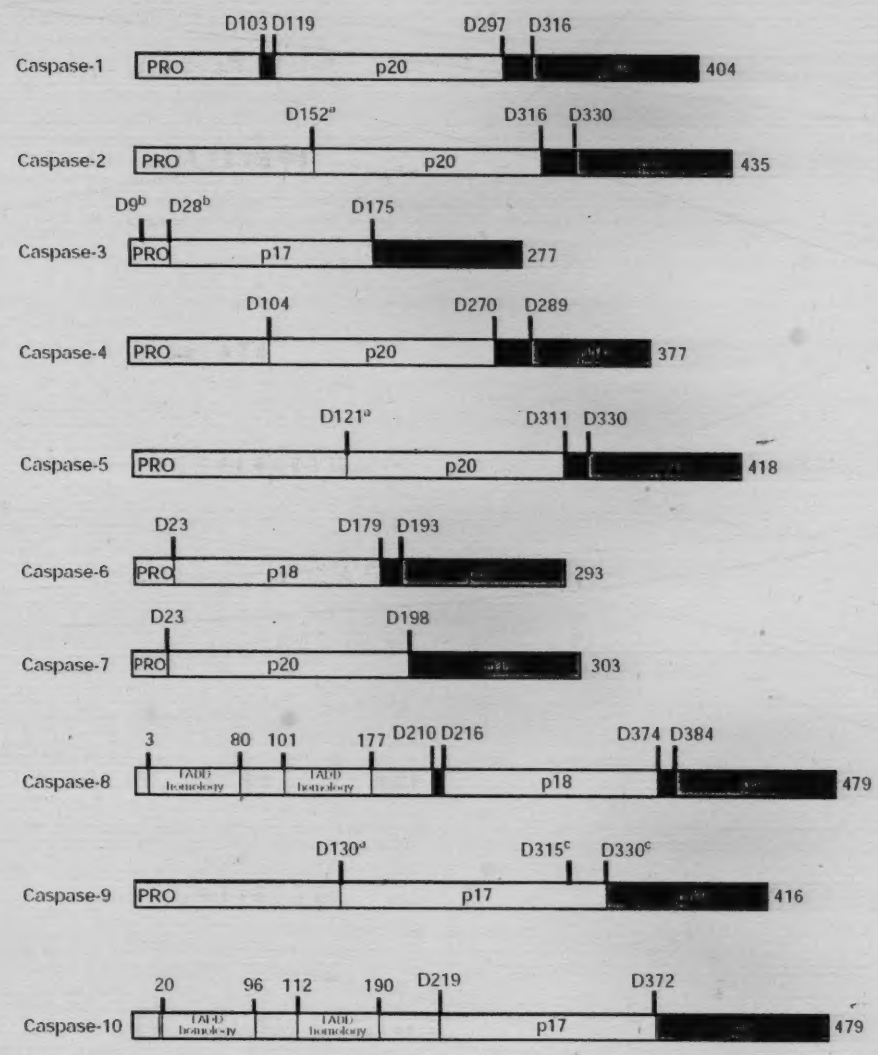


Figure 1.4: Molecular structures and cleavage sites of caspases. The procaspases consist of an N-terminal prodomain and two subunits, which separated by a linker region. The caspases are usually activated by cleavage after specific Asp residues, indicated by Dn (n is number of the amino acids in the protein). [a]Exact cleavage site not known. [b]Either of them (D9/D28) may be the cleavage site for caspase-3. Adapted from [59]

the procaspases to the specific death signaling complexes via adaptor molecules, resulting in their autocatalytic activation through a mechanism usually called "proximity-induced" activation [64]. Once activated, these initiator caspases can subsequently cleave and activate the downstream effector caspases - mainly caspase-3 [65]. The final targets of effector caspases include inhibitor of caspase-activated DNase/DNA fragmentation factor 45 (ICAD/DFF45), poly (ADP-ribose) polymerase (PARP), nuclear lamins, PAK2, as well as cytoskeletal proteins such as gelsolin and fodrin [66-68]. The cleavage and inactivation of ICAD/DFF45 results in the activation and translocation of CAD into nucleus and DNA fragmentation, which is seen as the characteristic 'DNA ladder' in apoptotic cells [66]. The cleavage of lamins confers the nuclear shrinking [67]. In addition, the caspase-mediated cleavage of gelsolin, fodrin, actin and PAK2 is responsible for the membrane blebbing and apoptotic body formation [68]. As a result, the cells will inevitably undergo completion of apoptosis.

1.3.1.2. Mechanisms of Caspase Activation

Thus far, there are three identified ways by which caspases can be activated [63]. One of the most clearly elucidated mechanisms is mediated by the death-inducing receptors, including Fas (CD95/Apo1), TNFR1/2, TRAIL-R (DR4/5), and DR3 (Wsl-1/Tramp) [63,69,70]. All these receptors contain a cysteine-enriched extracellular domain and an intracellular death domain (DD) [71]. Upon ligation by their corresponding ligands, the receptors trimerize and subsequently recruit the downstream adaptor molecules, such as FADD (Fas-associated death domain) for Fas [72]. FADD consists of an N-terminal DED motif and a C-terminal DD motif. Following binding to the trimerized Fas with its C-terminal DD, FADD can sequentially associate with

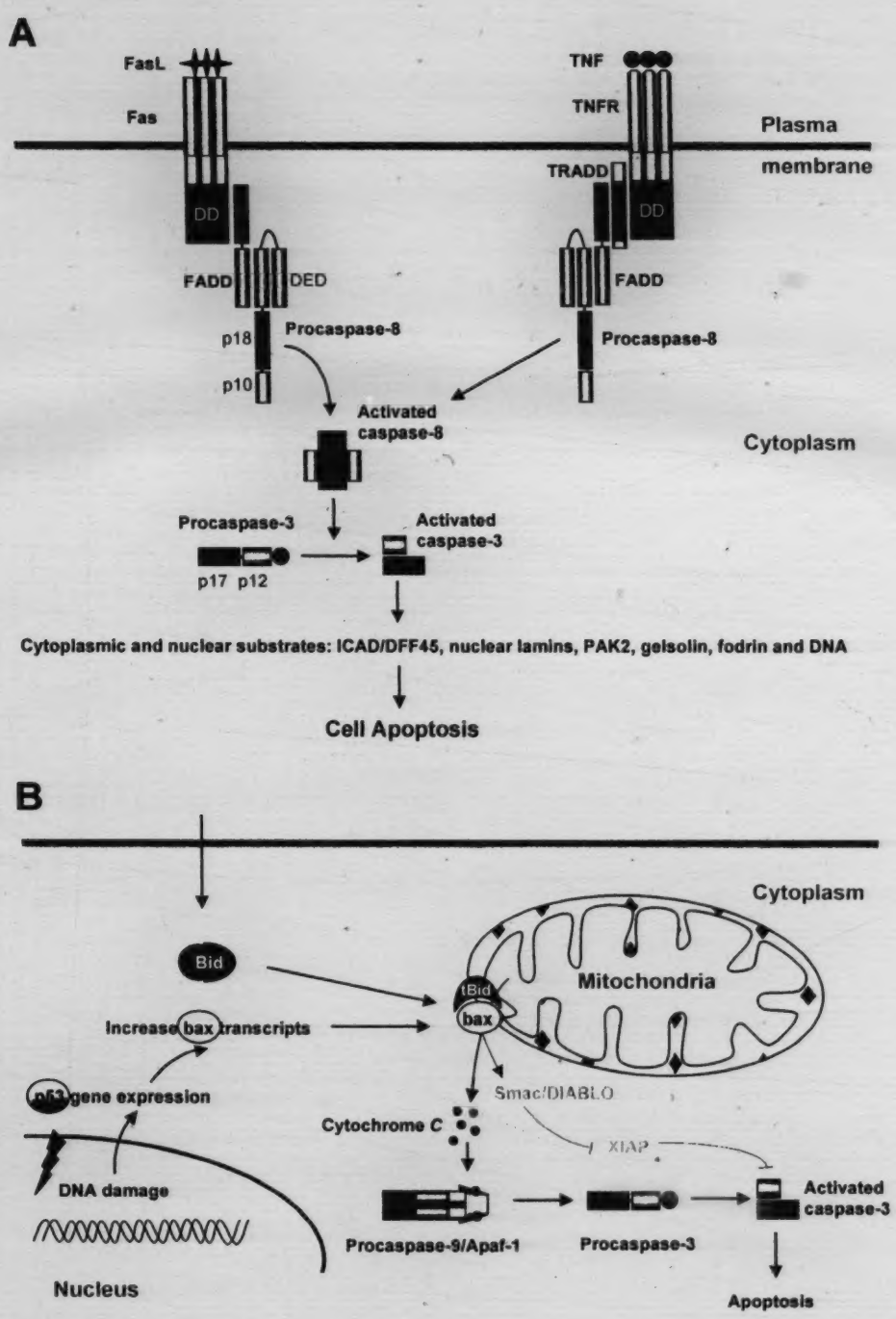


Figure 1.5: Mechanisms of caspase activation. Apoptotic caspase-cascade can be initiated through both (A) death receptor pathway and (B) mitochondrial pathway.

prodomain of caspase-8 through a DED-DED interaction (Figure 1.5 A) [73]. However, in the case of TNF-induced apoptosis, an additional scaffold protein TRADD (TNFR-associated death domain) is required for recruiting FADD to the signaling complex, so as to activate caspase-8 and the downstream effector caspase-3 (Figure 1.5 A) [74].

In addition to the death receptor-mediated caspase activation, intracellular stresses (e.g. DNA damage, nitrogen monoxide and radiation) can also initiate the caspase cascade through a mechanism involving the release of cytochrome *c* from mitochondria (Figure 1.5 B) [75]. In the cytoplasm, the cytochrome *c* forms a complex with Apaf-1 (apoptosis protease activating factor-1) and dATP, which subsequently binds and activates procaspase-9 to induce apoptosis through caspase-3 [18,76]. Other molecules released upon mitochondrial permeabilization include AIF (apoptosis inducing factor) and Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-associated binding protein with low pI), which can antagonize IAPs (inhibitor of apoptotic proteins) thereby promoting caspase activation [77,78].

Other than the two mechanisms described above, Granzyme B (GrB), a serine protease released by cytotoxic T cell, is evident to induce cell apoptosis by activating effector caspases such as caspase-3 [79,80].

1.3.2. TRAIL-induced Apoptosis

1.3.2.1. Therapeutic Role of TRAIL in Cancer

Recent studies have shown that targeting the death receptors of TNF family is a promising strategy for the treatment of solid cancers [81,82]. As described above, the death ligands (DLs) of the TNF family can induce apoptosis by binding their cognate death receptors (DRs), such as TNFα for TNFR1, Fas ligand (FasL, CD95L or Apo1L)

for Fas (CD95 or Apo1), Apo3L for DR3, and TRAIL (Apo2L) for DR4/DR5 [81]. It is well documented that the DL/DR interactions play crucial roles in the cytotoxic T-lymphocyte (CTL)-mediated and NK cell-mediated apoptosis of tumor cells [83-85]. Further, a recent study by Mehmut *et al.* suggests that the FasL- and TRAIL-mediated apoptotic signaling may confer the therapeutic effect of BCG instillation in TCCB [86]. However, despite the apoptotic effect of Fas and TNFα for cancer cells, their potential clinical usage was rapidly abandoned due to their significant toxicities to normal tissues [87,88]. As an alternative, since TRAIL by binding its receptors specifically triggers apoptosis of tumor cells, but spares normal cells [89,90], the therapeutic role of TRAIL-mediated apoptosis turned out to be one of the hottest areas in cancer research. Studies have shown that approximately 80% of human cancer cell lines are sensitive (at least to some extent) to TRAIL, whereas most normal cell types are relatively resistant [7,91]. In mice, TRAIL is shown to be effective at reducing mammary tumor growth without any of the toxic effects observed with the administration of FasL and TNF [91]. Furthermore, several studies have also shown a synergistic anti-tumor effectiveness when TRAIL is administrated as combination therapy with other anti-cancer drugs, such as doxorubicin, etoposide and AraC [92-94], highlighting the prospect of TRAIL as a reagent for future cancer therapy.

1.3.2.2. TRAIL and its Receptors

TRAIL, as a member of TNF family, is a type II transmembrane protein with a short intracellular amino-terminal tail and long extracellular carboxy-terminal portion, which was first identified and cloned based on its homology sequence with FasL/CD95L (28% identical) and TNF (23%) [9,10]. The extracellular domain of TRAIL forms a bell-

shaped structure of the receptor-binding site [11], and can be proteolytically cleaved from the cell surface by cysteine proteases to yield a vesicle-associated or a soluble, biologically active form of the ligand [95,96]. Similar to TNF ligands, crystal structure showed that TRAIL occurs as homotrimer (Figure 1.6 A and B) [11]. As a result, the binding of TRAIL results in its receptor trimerization and initiation of apoptotic signaling initiation (Figure 1.6 C and D) [97,98]. However, unlike other members of the TNF family whose expression is firmly regulated and limited in activated T cells, NK cells and immune-privileged sites, TRAIL and its receptors are ubiquitously expressed in most tissues and organs therefore the presence or absence of regulatory molecules is important in determining apoptotic sensitivity [9,10,99]. The distribution and translocation of the death and decoy receptors, as well as the relative ratio of pro- and anti-apoptotic proteins (e.g. Bcl-2 and c-FLIP), might cause the differential sensitivity of cells to TRAIL [93,100]. Also, the proapoptotic activity of TRAIL is dependent on the tertiary configurations of ligand/receptor complex. TRAIL has a free cysteine residue (Cys^{230}). The TRAIL containing free Cys-SH has higher affinity for the receptors and exhibits higher biological activity than the TRAIL modified by establishment of disulfide bridges [101].

TRAIL has been identified to bind four distinct cell surface receptors, including DR4 (TRAIL-R1) [102,103], DR5 (TRAIL-R2 or TRICK2/KILLER) [102,104,105], DcR1 (TRAIL-R3 or TRID/LIT) [104,106], and DcR2 (TRAIL-R4 or TRUNDD) (Figure 1.7 A) [107,108]. The fifth recognized TRAIL receptor is the soluble osteoprotegerin (OPG or DR5), which was initially identified as a receptor for RANKL/OPGL [109]. Since the affinity of OPG for TRAIL is very low, the physiological relevance of OPG as

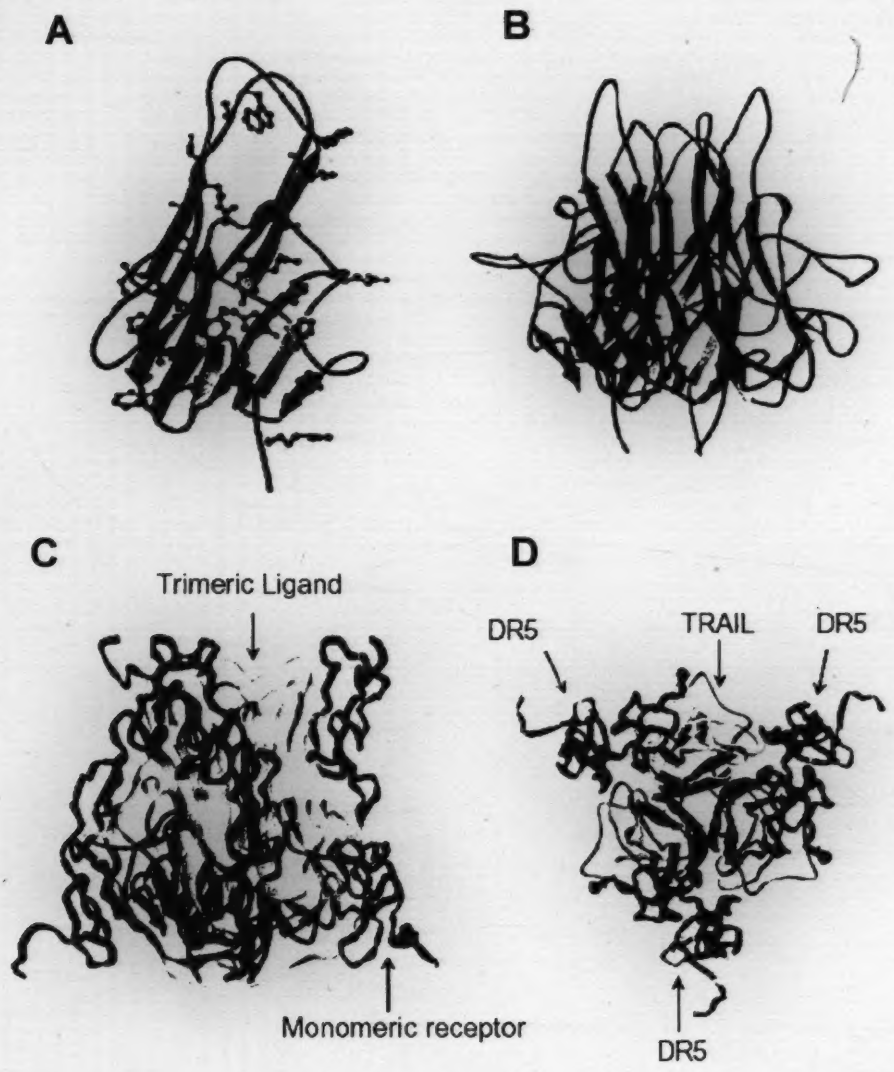


Figure 1.6: Crystal structures of TRAIL and death ligand/receptor complex. (A) Side view of TRAIL monomer. (B) Side view of TRAIL trimer. (C) Side view of the TRAIL/Death Receptor-5 (DR5) complex: the TRAIL trimer is encased in a clear shell, and the interdigitated DR5 monomers are indicated by red, green and blue colors. (D) Top view of the TRAIL/DR5 complex: the TRAIL monomers are indicated by yellow, pink, and turquoise colors. Adapted from [74].

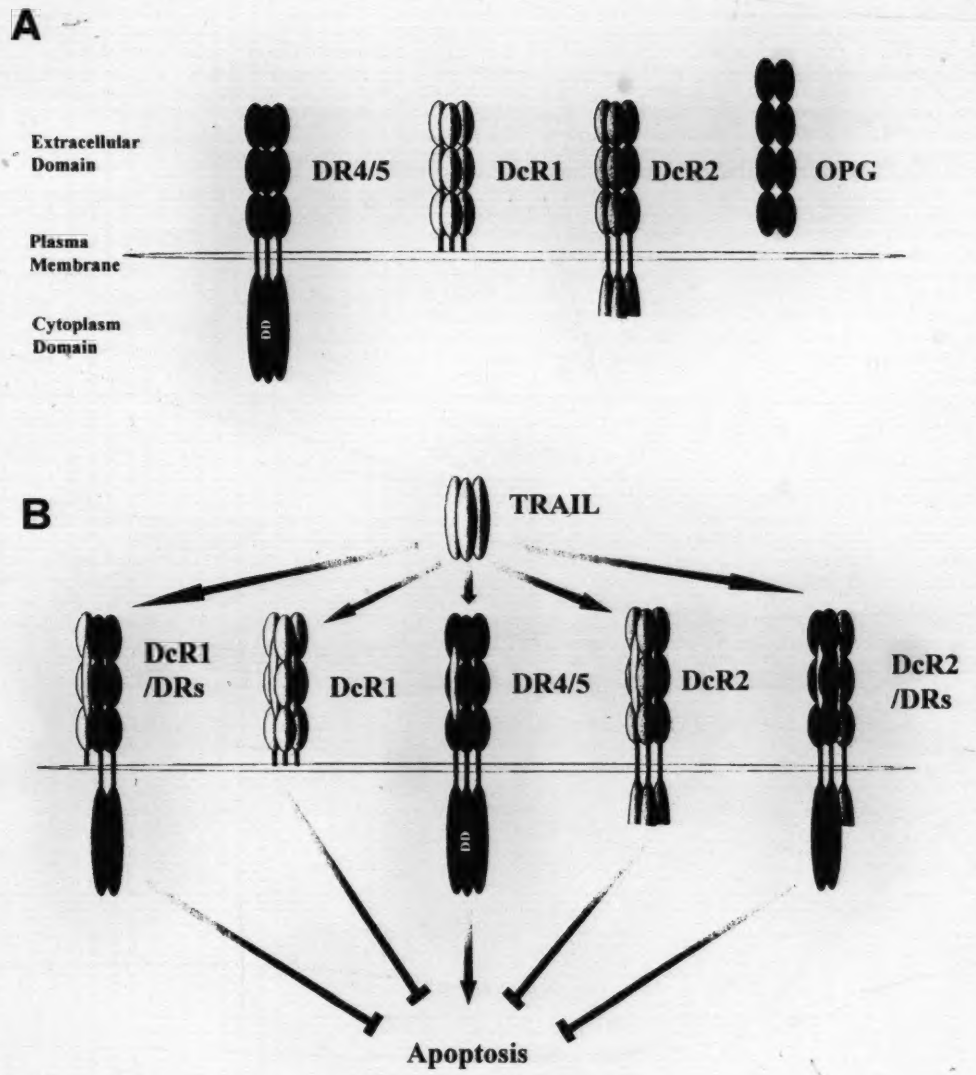


Figure 1.7: TRAIL receptors and biological activities. (A) Schematic structure of the TRAIL receptors. In contrast to other TRAIL receptors, the death receptors (DR4/5) not only contain the extracellular cysteine-rich domains (CRD, colored ovals), but also intracellular DDs. (B) Apoptotic signaling can be initiated after the binding of TRAIL to DR4/5. The decoy receptors (DcR1/2), as well as the mixed receptor complexes of both DcRs and DRs, can block the TRAIL-induced apoptotic signaling.

a receptor for TRAIL still remains unclear [110]. Structurally, all the membrane-associated receptors share highly conserved sequences in their extracellular domains. However, their intracellular domains exhibit wide diversities and are believed to confer their different biological activities following ligand binding.

The TRAIL death receptors, DR4 and DR5, are type I transmembrane proteins and expressed in wide range of tissues. In addition to the cysteine-rich domains (CRD) at extracellular domains, both of DR4 and DR5 contains an intracellular death domain (DD) capable of transmitting apoptotic signaling of TRAIL across the cell membrane (Figure 1.7 B) [102,103,111]. Recent studies suggested that these two receptors are regulated by p53 at transcriptional levels, and their apoptotic effects are enhanced in the presence of DNA-damaging therapeutics [112-114]. Further, it is evident that overexpression of these death receptors (e.g. DR5) by transient transfection can induce spontaneous apoptosis through clustering of the DDs [105]. Correspondingly, deletion of the DDs on these receptors results in the abrogation of TRAIL-induced apoptosis [102,103].

The TRAIL decoy receptors, DcR1 and DcR2, also contain extracellular TRAIL-binding potions. However, as compared with DR4 and DR5, both of them do not have functional DDs, since DcR1 lacks a cytoplasmic DD and DcR2 has a truncated DD by which no apoptotic signal can be transduced (Figure 1.7). Therefore they inhibit TRAIL-induced apoptosis as anti-apoptotic receptors by competing with DR4 and DR5 for binding to TRAIL [106-108,115,116]. DcR1 and DcR2 are generally expressed in normal tissues, but in a limited number on human tumor cells, which may be one of the reasons why normal cells are resistant to TRAIL [117,118].

OPG, as the only soluble TRAIL receptor is present mainly as a disulfide-linked homodimer [109]. Normally, it functions as a regulator in bone remodelling by competitively binding OPG ligand (OPGL/RANK), thereby preventing OPGL from binding to its receptor RANK on the osteoclasts and the subsequent osteoclastogenesis [119]. In parallel, it is generally believed that OPG is also serving as a decoy receptor for the TRAIL/DR-initiated apoptotic signaling [109,120].

1.3.2.3. TRAIL-induced Death Receptor Pathway

As previously described in section 1.3.1.2, there are two major mechanisms to activate caspases and initiate apoptosis, namely the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [63]. Both of these pathways are essential for the TRAIL-induced apoptosis (Figure 1.8). The earliest molecular events following the binding of TRAIL to DR4/DR5 are the receptor trimerization and clustering of the intracellular DDs of the DRs, which lead to the construction of DISC (death-inducing signaling complex) [14,121]. This is achieved by sequential recruitment of the adaptor molecule FADD (Fas-associated death domain) and caspase-8 [13]. FADD has a carboxy-terminal DD and an amino-terminal death effector domain (DED) [122,123]. Therefore, through the homophilic DD-DD interaction, FADD interacts with the cytoplasmic domain of DR4/DR5. Further, via DED-DED interaction FADD recruits apoptosis-initiating caspase-8, that contains two DED and one caspase domain, to the receptor-associated DISC [13,124,125]. Oligomerization of caspase-8 in the DISC leads to its conformational changes and autocatalytic activation through two consecutive auto-cleavage steps [14]. The initial cleavage at Asp374 generates an N-terminal p43 and a C-terminal p12 fragment [14]. The p12 intermediate is then further cleaved generating a

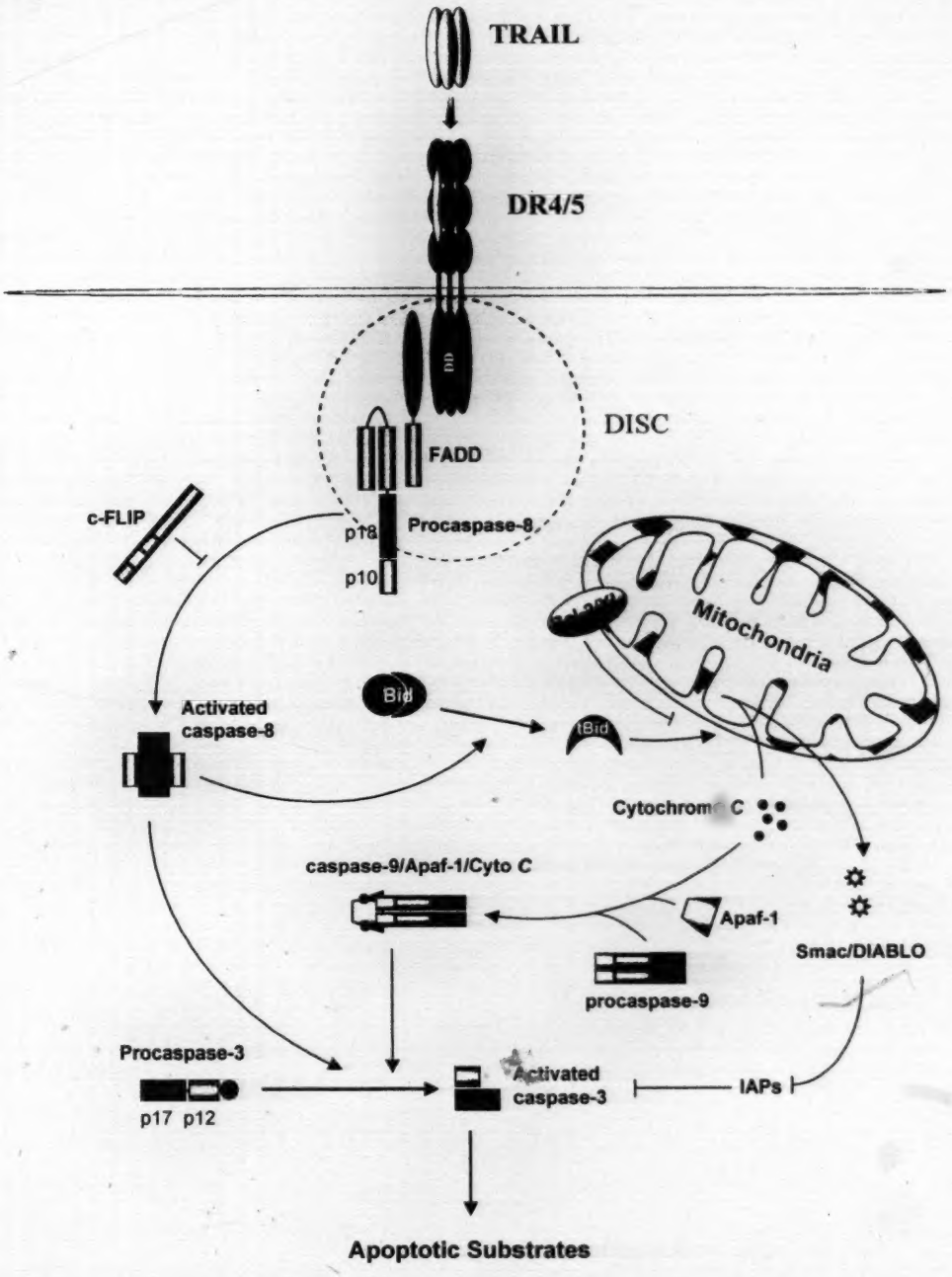


Figure 1.8: TRAIL-induced apoptotic signaling. Binding of TRAIL to its death receptors (DR4/DR5) initiates DISC formation and caspase-8→caspase-3 cascade. By caspase-8-mediated Bid cleavage, TRAIL also induces apoptosis through mitochondrial pathway.

p10 fragment. As well, the subsequent process of the receptor-bound p43 intermediate generates the p26 prodomain and the p18 fragment containing the QACQG-active site [14]. As a result, the cleavage-activated p18 – p10 fragments of caspase-8 are dimerized and released into the cytosol where they activate the downstream effector, caspase-3 [73,126,127]. The mature form of activated caspase-3 is composed of two subunits of p17 and p12, which are generated by cleavage of the proenzyme at Asp28 and Asp175 [128]. Some studies also suggest that the initial cleavage may occur at Asp9 instead of Asp28 [129,130]. During the execution phase of apoptosis, caspase-3 is the central molecule that is responsible for most of the catalytic process on substrates containing Asp-Xaa-Xaa-Asp (DXXD) motif, such as DNA fragmentation factor 45 (DFF45), directly resulting in cell apoptosis [16]. (See details in Figure 1.8)

Although FADD and caspase-8 are required for the TRAIL-induced apoptotic signaling, the activation of the downstream pathways of caspase-8 varies within different cell types. It is evident that, in some lymphoid cell lines, caspase-8 is dynamically activated following DL/DR interaction and directly activates downstream caspase-3 [131]. However, in some circumstances, caspase-8 activation is not enough to directly or fully activate caspase-3 [131-134]. As an alternative, the caspase-8-mediated truncation of Bid can significantly increase its apoptotic activity through mitochondrial pathway [135].

1.3.2.4. tBid-mediated Mitochondrial Pathway

The cross-talk between the death receptor pathway and the mitochondrial pathway is mediated by Bid, a member of the "BH3 domain only" subgroup of the Bcl-2 family [135]. Normally, this set of pro-apoptotic proteins appear to exist as inactive conformations and contain conserved sequence within the BH3 amphipathic α helical

domain, which is essential for heterodimerization with other Bcl-2 family members and apoptotic activity [136]. Upon activation by death receptor engagement, caspase-8 can subsequently cleave and activate the cytosolic p22 Bid to generate truncated p15 Bid (tBid) [135,137]. As a result, tBid translocates to the mitochondrial membrane and induces the release of apoptotic factors such as cytochrome *c* and Smac/DIABLO [17,138]. The Smac/DIABLO protein can neutralize the anti-apoptotic activity of IAP (inhibitor of apoptosis protein) that functions to antagonize the activation and activity of caspase-3, thereby promoting apoptosis [77,78]. Most significantly, as previously described in section 1.3.1.2, the cytosolic released cytochrome *c* can bind to Apaf1 and then associates with caspase-9 to form the apoptosome where caspase-9 is activated and in turn cleaves caspase-3 to induce apoptosis [18]. (See details in Figure 1.8)

Although the exact mechanism(s) by which cytochrome *c* is released from mitochondria is still obscure, two main theories have been put forward depending on the different cell types and study models. Research by Schlesinger *et al.* supports that the activated tBid serves as a "death ligand" for the mitochondria resident proteins, such as pro-apoptotic Bak and Bax [139]. Binding of tBid induces the intramembranous oligomerization of these apoptotic proteins into a proposed pore on mitochondria, resulting in cytochrome *c* release [139]. Alternatively, it is also proposed that tBid by inserting itself into mitochondrial membrane and forming intramembranous pores can directly induce cytochrome *c* release as an effector protein [140]. However, despite the ambiguity on how tBid induces cytochrome *c* release and activates the mitochondrial pathway, the absolute necessity of tBid in mitochondrial pathway is further entrenched in loss-of-function approaches including gene knockout and immunodepletion [137,141].

In addition to tBid, other Bcl-2 family members also play vital roles in the mitochondrial pathway [142]. Contrary to the pro-apoptotic members, such as Bax and Bak that usually display less sequence conservation of the BH4 domain [143], Bcl-2 and Bcl-X_L function as anti-apoptotic proteins to inhibit mitochondrial release of the apoptotic factors (Figure 1.9) [144]. These anti-apoptotic members and pro-apoptotic members of Bcl-2 family meet at the surface of mitochondria, where they compete to regulate cytochrome *c* release by a mechanism that is still debated. Of particular significance, accumulating evidence suggests that the anti-apoptotic Bcl-2 is frequently upregulated in bladder cancers and correlated with the resistance of tumor cells to apoptosis [145-149].

1.3.3. Anti-apoptotic Proteins

1.3.3.1. Bcl-2

The *Bcl-2* gene was initially recognized as a proto-oncogene translocated to the immumoglobulin heavy chain locus (t 14;18) in human B cell lymphomas [150,151]. It is the archetype of a novel class of oncogenes that contributes to neoplastic cell growth by enhancing cell survival through the inhibition of apoptosis, instead of accelerating the rate of cellular proliferation. Structurally, Bcl-2 consists of four BH domains (BH1-BH4) and a carboxy-terminal hydrophobic tail, which facilitates the localization of the protein to the cytoplasmic surface of mitochondria. Thus far, several possible mechanisms have been proposed to explain the anti-apoptotic activity of Bcl-2 protein: (i) protection of mitochondrial integrity and thereby keeping enclosed a plethora of pro-apoptotic molecules including cytochrome *c*, DIABLO/Smac, and AIF; (ii) sequestration of caspase-cascade by directly binding the adaptor protein (e.g. Apaf-1 homologue CED-4)

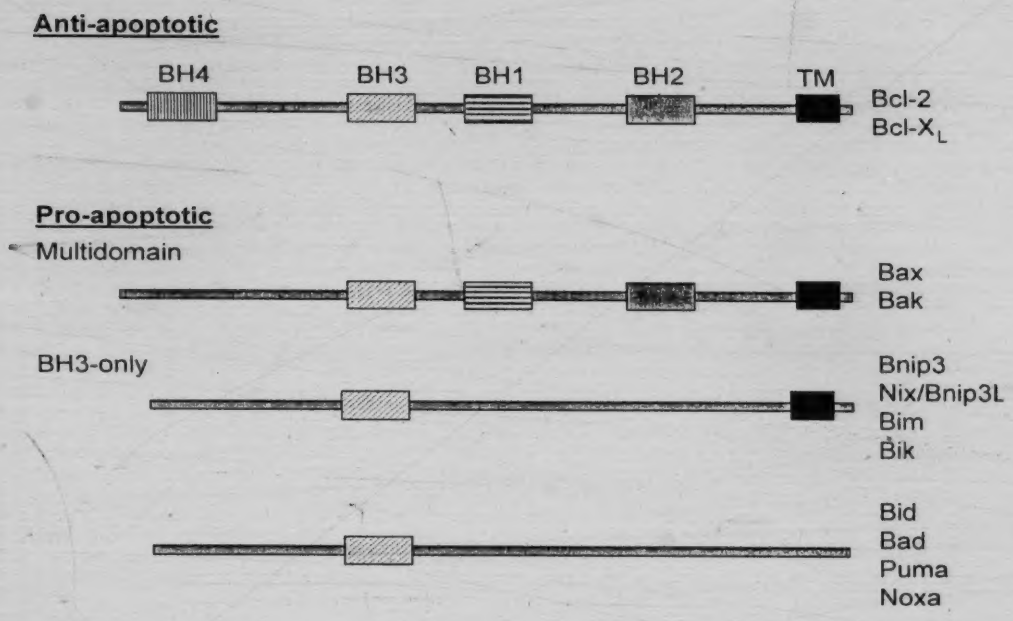


Figure 1.9: Three subgroups of the Bcl-2 family proteins. The Bcl-2 family proteins contain one or more of the Bcl-2 homology (BH) domains (i.e. BH1, BH2, BH3 and BH4). Most members also have a C-terminal transmembrane domain (TM). The anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, conserve all four BH domains. Further, the pro-apoptotic family members can be subdivided into those have multidomains (e.g. Bax and Bak) and the proteins that have only BH3 domain (e.g. Bid and Bad). It is generally proposed that the multidomain pro-apoptotic members can initiate permeabilization of mitochondrial membrane and release of apoptotic molecules (e.g. cytochrome *c* and Smac/DIABLO), which can be inhibited by anti-apoptotic Bcl-2 family members. The BH3-only Bcl-2 family proteins are believed to trigger mitochondrial permeabilization indirectly by binding anti-apoptotic members, thereby releasing the multidomain pro-apoptotic members to facilitate the apoptotic process. Adapted from Gustafsson and Gottlieb, Am J Physiol – Cell Physiology, 2007.

to prevent it from activating the downstream caspase (caspase-9 homologue CED-3); (iii) Regulation of the activation of initiator caspases that act upstream or independently of the mitochondrial pathway (Figure 1.10) [152-155]. Although none of these mechanisms have been proven definitely, it is well documented that overexpression of Bcl-2 blocks mitochondrial pathway to apoptosis.

Moreover, Thomas *et al.* using melanoma cells showed that overexpression of Bcl-2 blocks TRAIL-induced apoptosis [156]. Supportive of this, Bcl-2 overexpression has also been shown to block TRAIL-induced apoptosis in human lung cancer cells and this correlated with inability of cytochrome *c* release and PARP cleavage [157]. Taken together, these results strongly signify the dependency of TRAIL-induced apoptosis on the mitochondrial pathway and the anti-apoptotic role of Bcl-2 in this process. Further, the clinical significance of Bcl-2 overexpression was also demonstrated in bladder cancers, where it correlated with superficial TCCB recurrence and progression [145,147], poor prognosis of invasive cancer [158-160], as well as being a factor in patients response to chemotherapy or radiotherapy [146,149]. Recently, accumulating evidence suggest that downregulation of Bcl-2 may potentiate TNF- and chemotherapeutic reagent-mediated cell death [161-163]. This is consistent with the previous studies by Miyake *et al.* (1998) showing that transfection of Bcl-2 into the human bladder KoTCC-1 cell line, which does not have endogenous Bcl-2 protein, significantly desensitize the transfected cells to cisplatin (one of the most effective chemotherapeutic agents for TCCB) as compared with their parental KoTCC-1 cells [148]. Therefore, the approach to inhibit or decrease the expression of Bcl-2 is becoming obligatory as a novel therapy in the future TCCB treatment.

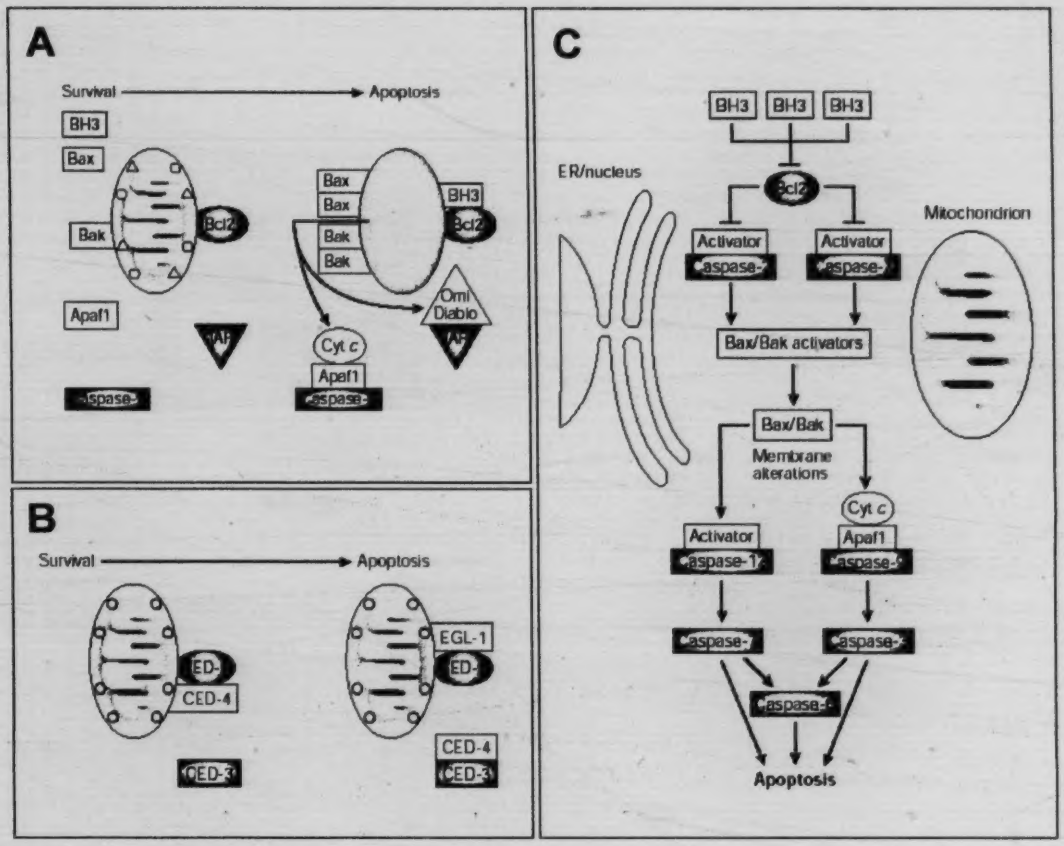


Figure 1.10: Possible anti-apoptotic mechanisms of Bcl-2. (A) Bcl-2 protects the mitochondrial integrity and prevents cytochrome *c*/Smac-DIABLO release by neutralizing pro-apoptotic Bcl-2 family members. (B) In *Caenorhabditis elegans*, caspase-9 homologue CED-3 by sequestrating the adaptor protein CED-4, prevent it from activating the downstream caspase. (C) In a hypothetical model, Bcl-2 by sequestrating the activators of upstream initiator caspases at mitochondria and endoplasmic reticulum.

1.3.3.2. Clusterin

Clusterin is another protein that correlates with tumor progression and recurrence when it is overexpressed in TCCB [164]. Clusterin was initially recognized as a secreted glycoprotein and characterized as a cell-aggregating factor *in vitro*; hence the name Clusterin [165]. Based on the source of purification (or gene cloning), it was also named CLI (complement cytolysis inhibitor), SP-40, 40 (secreted protein 40, 40) and ApoJ (due to similarities with other apolipoproteins) [166-168]. Although intensive studies have been conducted during the past decades, Clusterin still remains enigmatic because of its multi-functionalities in different cell types and tissues. In 2000, convincing evidence provided by Poon *et al.* demonstrated that Clusterin has chaperone-like activity, similar to that of small heat shock proteins (sHSPs), as it can bind and stabilize stressed proteins in a folding-competent state *in vitro* [169]. Normally, HSPs are produced in response to different types of stress conditions, thereby making cells resistant to stress-induced cell damage through their chaperone functions. For example, HSP27 is known to exert a clear cytoprotective effect against a large variety of apoptosis-inducing agents by inhibition of apoptosome formation [170,171]. As well, Clusterin has been reported to protect various cell types from TNF-α and doxorobucin (DXR)-mediated cytotoxicity extracellularly [172,173]. Based on these observations, together with the panel of extracellular proteins bound to Clusterin and the existence of the HSF1 responsive element in the Clusterin gene promoter, it is believed that Clusterin could function as an extracellular chaperone [174]. But on the other hand, Clusterin was also reported to exacerbate oxygen or glucose-deprivation-induced cell necrotic death and brain injury with a mechanism(s) that is still elusive [175]. In addition to the secreted form of

Clusterin, several other intracellular isoforms of Clusterin have been reported. A high mannose form of Clusterin of ~60 kDa that is not internally cleaved has been identified in the cytoplasm of several malignancies and senescent cells [176,177]. A 43 kDa non-glycosylated Clusterin form has also been reported in some cases of advanced breast cancers that functions intracellularly as a molecular adaptor [178,179]. Most recently, Zhang *et al.* reported that the intracellular Clusterin, in response to chemotherapeutic agents, could impede Bax oligomerization on mitochondrial surface and thereby prevent the release of cytochrome *c* and mitochondrial apoptotic pathway [180]. Taken together, these findings strongly suggest that Clusterin could be implicated in a wide range of biological activities from cell adhesion, DNA repair to live/death signal transduction that largely depends on its extra/intracellular localization, the cell/tissue type as well as the kind of ongoing stimuli [181,182].

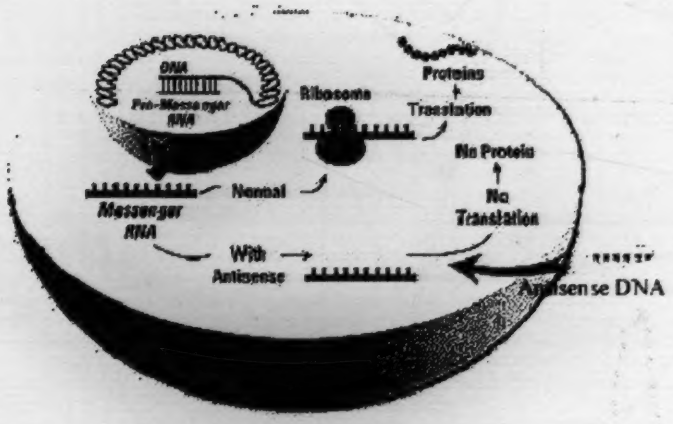
Clinically, Clusterin is frequently overexpressed in various human malignancies, where it correlates with tumor progression [183-185] and resistance to cancer therapies [186]. Moreover, overexpression of Clusterin in senescent cells may represent a predisposition to carcinogenesis [187]. Although the exact role of Clusterin as a pro- or anti-apoptotic molecule is still a matter of ambiguity due to the contradicting reports, the upregulation of Clusterin mRNA and protein levels is a common phenomenon in developmental and pathophysiological states, suggesting a crucial therapeutic effect of controlling Clusterin expression levels. In particular, Miyake *et al.* have used antisense Clusterin oligonucleotide therapy to downregulate the protein level of Clusterin and found that it could significantly enhance the cytotoxic effect of cisplatin on the cellular growth and metastasis of human bladder cancer KoTCC-1 cells *in vitro and in vivo* [188].

1.4. Antisense Oligodeoxynucleotides

Antisense oligodeoxynucleotides (ASO) refer to short, synthetic oligodeoxynucleotides (ODN) that can be as short as 15 mers, and have the required specificity to inhibit particular gene expression by annealing to the cellular mRNA [189,190]. The stability of the ODN-mRNA duplex in terms of hybridization and half-life is critical to successful gene inhibition. The mechanism of inhibiting the gene expression is based on two properties. The first is the physical blocking (ribosomal blockade) of the translation process due to the existence of the short double stranded region on mRNA, which prevents the ribosome from reading it. The second is the cellular RNase H activity on the ODN-mRNA duplex, which cleaves the RNA-ODN duplex region of the mRNA thus inhibiting protein synthesis (Figure 1.11 A) [191].

The initial development of phosphorodiester ASO provided a way to recruit RNase H and instigate its hydrolytic activity on the ODN/mRNA hybrids. However, the phosphorodiester ASO are sensitive to nucleases and have relatively short half-lives [192]. Although they are still in use, most current applications rely on the employment of chemically modified ODN. Numerous studies on nucleic acid chemistry have been dedicated in developing novel base analogues that are not only resistant to degradation but also possess strong hybridization properties. Ongoing chemical modification of ASO aims to (i) improve the stability and affinity of ASO to target mRNA, (ii) facilitate recruitment of RNase H to cleave targeted mRNA efficiently, and (iii) reduce the toxicity of ASO. Methylphosphonate analogues, as one of the earliest modifications, are generated by replacing the non-bridging oxygen at phosphorus in the ODN backbone with a methyl (CH_3) group (Figure 1.11 B). Although the methylphosphonate ASO is

A



B

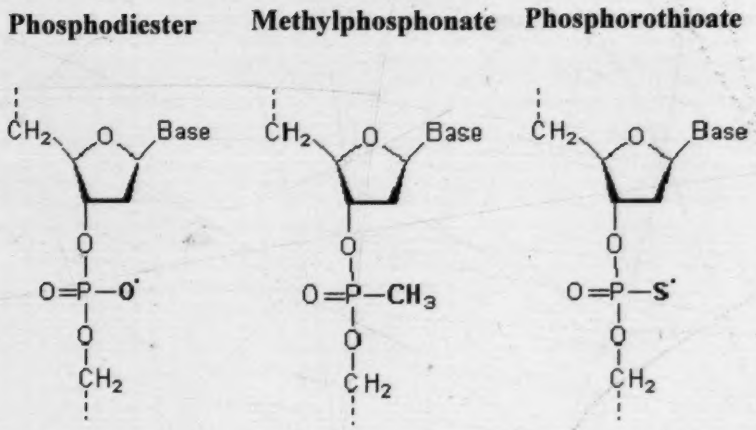


Figure 1.11: Working mechanism of antisense oligodeoxynucleotides. (A) Antisense oligodeoxynucleotides inhibit gene expression by physical blocking translational process and initiating cellular RNase H activity on ODN-mRNA duplex. (B) Molecular structures of chemically modified ODN. Adapted from http://www.cem.msu.edu/~cem181h/projects /97/antisense/index.html

quite resistant to nucleases, its capability to hybridize to complementary RNA is rather weak and it is incompetent of directing RNase H activity [193,194]. Currently, the most frequently used chemically-modified ASO is phosphorothioate-linked ODN whose sulfur (S-) group is the replacement for the unbridged oxygen of phosphorodiester ASO (Figure 1.11 B). Phosphorothioate ASO are not only able to resist nuclease-mediated degradation and thus have a long half-life *in vitro* and *in vivo*, but also maintain the ability to recruit RNase H in order to degrade ODN-mRNA hybrids [195,196].

When applied on cells, naked ASOs usually do not permeate across the plasma membrane. Endocytosis of a small fraction may happen but the chance is very low considering the poor adsorption between the negatively charged ODN and the negatively charged plasma membranes. In addition, the presence of the nucleotide bases in the ASO makes oligodeoxynucleotides too polarized for passive diffusion [197]. The vector-facilitated delivery of ODN, through the use of liposomes, polymers or peptides, appears to be an appropriate and efficient way to overcome the plasma membrane barrier. Cationic liposomes are capable of forming stable complexes with the negatively charged ODNs, since they mainly consist of positive charged lipids. Using these agents, such as lipofectin, a considerable enhancement of the ODN adsorption via membrane can be obtained [198]. Some membrane proteins have also been reported to be involved in liposome-mediated delivery of oligonucleotides by triggering endocytosis [199,200].

The potential cytotoxicity of the ASO (e.g. phosphorothioate-linked ODN) is its affinity for membrane and other intracellular proteins, although this affinity may protects them from being removed by filtration *in vivo* and prolongs the circulation time of ODN [201,202]. For instance, the phosphorothioate ODN may bind in a length manner and to

some extent in a sequence-dependent manner to heparin-binding proteins, such as the epidermal growth factor receptor (EGFR), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and its receptor as well [203]. Normally, the *in vivo* toxicity correlates with the capture and long-term deposit of ODN in reticuloendothelial system (RES). Organs, such as liver, spleen, kidney and lungs are primarily affected following systemic administration of ODN complex [204,205]. Nevertheless, with non-systemic intravesical treatment for bladder cancer for a short time, the cytotoxicity may not be a big concern. Also, the cytotoxicity can usually be controlled through adjusting the proper dosage of ODN or their packaging into delivery vectors *in vitro*. Phosphorothioate ASO have been used successfully in a large number of experiments designed to downregulate gene expressions, as well as in several clinical therapeutic trials [206-210]. In bladder cancer, Bilim *et al.* have examined ASO-mediated downregulation of Bcl-2 expression and its effect on sensitivity to Adriamycin in T24 cells and found that ASO successfully reduce the protein level of Bcl-2 and resulted in a synergistic cytotoxicity with Adriamycin [211]. Thus far, the potential toxicity of chemically modified ODN in intravesical therapy has not been addressed. Basing the clinical toxicity profile of ODN directed against viruses and tumors, this new generation of drugs is still more tolerable than conventional chemotherapy and radiotherapy [212-214].

1.5. Rationale and Hypothesis

Transitional cell carcinoma of the bladder (TCCB), as the second most common urologic malignancy, is usually superficial and treated by transurethral resection with subsequent intravesical therapies, which include chemotherapies and immunotherapies

(e.g. BCG). But as described above, both intravesical therapies and systemic chemotherapies have side effects of various degrees [34,49,50]. Moreover, TCCB ultimately fails treatment because the cells are resistant to apoptotic induction. All of the evidence above supports development of newer combination strategies that are more selective and less toxic.

TRAIL (tumor necrosis factor related apoptosis-inducing ligand) has long been a promising candidate for novel cancer therapy, since it can selectively trigger apoptosis in cancer cells but spare normal cells [89,90]. However, up to date, little is known is about the apoptotic effect of TRAIL as a potential anti-tumor reagent in TCCB treatment. It is well documented that TRAIL can induce apoptosis through both death receptor pathway and mitochondrial pathway [63]. Recently, accumulating evidence suggest that the tBid-mediated mitochondrial pathway is critical for TRAIL-induced apoptosis in various cancers, and Bcl-2 overexpression is responsible for the desensitization of apoptotic signal in most of cases [156,157]. Intriguingly, overexpression of Bcl-2 and Clusterin is frequently observed in bladder cancers, where it correlated with tumor recurrence and progression, poor prognosis, and resistance of chemotherapy or radiotherapy [145-149,158-160]. To investigate potential therapeutic role of TRAIL for TCCB cells, the well established ASO technology provides us an efficient tool to evaluate the possible potentiating effect of downregulation of the anti-apoptotic proteins, such as Bcl-2 and/or Clusterin, on TRAIL-induced apoptosis of TCCB cells.

In this study, we hypothesized that **TRAIL promotes apoptosis and clearance of TCCB with minimal toxicity and ASO Bcl-2 and/or Clusterin will enhance this process**. To examine this proposition, three specific aims were proposed as following:

Aim 1: To investigate the sensitivity of TCCB cells to TRAIL-induced apoptosis and the TRAIL-induced death receptor and mitochondrial apoptotic signaling in TCCB cells

Aim 2: To downregulate the expression of Bcl-2 and/or Clusterin by ASO

Aim 3: To examine the effect of Bcl-2 ASO and/or Clusterin ASO on TRAIL-induced apoptosis.

Chapter 2: Materials and Methods

2.1. Reagents

Recombinant non-tagged soluble human TRAIL (amino acid 114-281) was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA), and then prepared as 1 μg/μl stock solution in sterile distilled water and stored at -20°C for further use. DMEM culture medium, MEM (supplemented with L-Glu NEAA Earle's BSS), RPMI-1640, McCoy's 5A medium, Opti-MEM I serum-reduced medium, 100X antibiotic-antimycotic, lipofectin transfection reagent, ethylenediaminetetraacetic acid (EDTA), pheylmethysulfonyl fluoride (PMSF), and trypsin were purchased from GIBCO Invitrogen, Inc (Carlsbad, CA, USA). The antisense oligodeoxynucleotides specific for Bcl-2 (Bcl-2 ASO: AAGGCATCCCAGCCTCCGTT) and Clusterin (Clusterin ASO: CAGCAGCAGAGTCTTCATCAT), as well as the mismatched sequence (MM: CAGCAGCAGAGTATTTATCAT), were kindly provided by Dr. Martin Gleave (University of British Columbia, BC, Canada). Fetal bovine serum (FBS) was purchased from North Bio, Inc (Toronto, ON, Canada). Protease inhibitor cocktail, N,N,N',N'-Tetramethylethylenediamine (TEMED), Trizma hydrochloride (Tris-HCl), Tris-Base, polyoxyethylene-sorbitan monolaurate (Tween-20), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), dithiothreitol (DTT), and MTT, i.e. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, were purchased from Sigma-Aldrich, Inc (Oakville, ON, Canada). Dimethyl sulfoxide (DMSO), glycine, glycerol, sodium chloride (NaCl), ammonium persulfate (AP), methanol, 30% (w/v) acrylamide stock solution, and Triton X-100 were purchased from Fisher Scientific (Nepean, ON, Canada). Bromophenol blue, dual color protein standard and nitrocellulose membrane were purchased from Bio-Rad Laboratories Ltd (Hercules, CA, USA). BCA™ Protein

Assay Kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Enhanced chemiluminescence (ECL) and ECL-plus Western Blotting detection reagents were purchased from Amersham Biosciences (England, UK). SYTO-16 cell-permeant nucleic acid stain and propidium iodide (PI) non-permeant nucleic acid stain were purchased from Molecular Probes (Eugene OR, USA).

2.2. Antibodies

Rabbit polyclonal antibodies against human caspase-3, caspase-9, and Bid were purchased from Cell Signaling, Inc (Danvers, MA, USA). The mouse anti-caspase 8 (FLICE) antibody was purchased from Medical & Biological Laboratories Co., LTD (Woburn, MA, USA). Goat polyclonal antibody against human Clusterin (C-18) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Mouse monoclonal antibody against human Bcl-2 (clone 100) was purchased from Upstate Cell Signaling (Temecula, CA, USA). The mouse anti-DFF45/ICAD monoclonal antibody was obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Mouse monoclonal antibody against β-tubulin was purchased from Sigma- Aldrich, Inc (Saint Louis, MO, USA). The goat anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies, as well as the Rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody, were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA, USA).

2.3. Cell Culture

Human TCCB cell line CRL-1472 (HT-1376) and HTB-9 (5637) were purchased from American Type Culture Collection (ATCC). TCCB cell line UMUC3, UMUC6,

UMUC9, and UMUC14 were a generous gift from Dr. H. Barton Grossman (MD Anderson Cancer Center, TX, USA). TCCB cell line RT-4, 253J and T24 (EJ28) were kindly provided by Dr. Martin Gleave (University of British Columbia, BC, Canada). TCCB cell line RT-112 and MGH-U3 were generously provided by Dr. Yves Fradet (Universite Laval, QC, Canada). The normal fibroblast F2P6 cells were obtained from ATCC. CRL-1472 (HT-1376), UMUC6 and UMUC14 were maintained in MEM supplemented with 10% FBS and 1X antibiotic-antimycotic. HTB-9 (5637), T24 (EJ28), 253J, RT-112 and MGH-U3 were maintained in RPMI-1640 supplemented with 10% FBS and 1X antibiotic-antimycotic. RT-4 was maintained in McCoy's 5A supplemented with 10% FBS and 1X antibiotic-antimycotic. UMUC3, UMUC9 and the normal fibroblast cell line F2P6 were cultured in the DMEM supplemented with 10% FBS and 1X antibiotic-antimycotic. All the culture media were renewed every 2 to 3 days. To subculture cells, the culture medium was discarded and the cell monolayer was then rinsed once with 1X phosphate-buffered saline (PBS) to remove all traces of serum that would neutralize Trypsin. Sequentially, 5ml of 0.5X Trypsin-EDTA in HBSS solution was added to each T75 cell culture flask for 3-5 minutes until the cell layer was detached from the bottom of the flask. Then, 5ml of culture media containing 10% FBS was added to inhibit the activity of Trypsin. After cell suspensions were centrifuged at 1000rpm for 3 minutes (Model J-6 Centrifuge, Beckman Inc. Mississauga, ON, Canada), cells were re-suspended with the complete culture media. Appropriate aliquots of the cell suspension were transferred to new culture flasks. All cell lines are cultured at 37°C in humidified incubator containing 5% CO_2 (Water-Jacketed Incubator, Forma Scientific, Marietta, OH, USA).

2.4. MTT Assay

Proposed cells were seeded in the flat-bottom 96-well plates (Corning) at ~1 x 10^4/well with 100µl growth media and cultured overnight at 37°C, 5% CO_2. Then, the cells were subjected to by corresponding drug treatments. After that, the cells were washed once with PBS and incubated with 50µl of MTT solution (60µg/ml in colorless RPMI-1460 media) for 4 hours at 37°C. 150µl of DMSO was then added in to dissolve the formazan crystals which were thoroughly mixed into the solvent by shaking the plates (vortex 12-812, Fisher Scientific, Nepean, ON, Canada) at ~150rpm for 20 minutes. The number of surviving cells is directly proportional to the level of the formazan product created. The absorbance of the formazan product was determined at 570 nm with the 655nm filter as reference. After normalization to the absorbance value of untreated cells, the percentage of cell death was obtained through the equation: (1-optical density of cells treated / optical density of cells untreated) x 100.

2.5. Protein Extraction and Protein Assay

Following the treatments under each experimental condition, cells were rinsed twice with ice-cold PBS and then scraped off the culture dishes into ice-cold lysis buffer (20mM Tris–HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 2mM EDTA, 10% glycerol, freshly supplemented with 0.5% (v/v) protease inhibitor cocktail and 1mM PMSF). Then, the cell suspension was transferred to the pre-marked tubes and homogenized with vigorous pipetting. After sitting on ice for 20 minutes, cell suspensions were centrifuged at 14000 rpm for 15 minutes at 4°C (EBA12 Hettich Zentrifugen, Rose Scientific Ltd,

Edmonton, AB, Canada). The resulting supernatants were subsequently assessed for protein concentration using BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), mixed with 3X Laemmli Sample Buffer (18.8% 1M Tris-HCl pH 6.8, 6% SDS, 4.7% DTT, 30% glycerol, and 0.15% Bromophenol blue) and boiled for 5 minutes. The resultant samples were then either stored at -20°C or subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.6. SDS-PAGE and Western Blotting

Using Bio-Rad Mini-PROTEAN III system, electrophoresis was performed on 15% resolving gels with 5% stacking gels (Table 2.1). Equal amounts of protein samples were separated at 100V (constant voltage) for the initial 15 minutes followed by 150V (constant voltage) for the remaining 70 minutes in 1X Running Buffer (Table 2.1). The separated proteins were then transferred to a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot system for 980 minutes at 30V (constant voltage) in 1X Transfer Buffer (Table 2.1). Subsequent to transferring, the membranes were blocked in 5% (w/v) non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 2 hour at room temperature (RT) or overnight at 4°C. The membranes were then incubated with primary antibodies of known specificity against the proteins of interests overnight at 4°C, at concentrations recommended by manufacturers or other providers. This was followed by three successive washes (5, 5, 10 minutes) in TBS-T. Sequentially, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit or rabbit anti-mouse secondary antibodies in 5% milk (in TBS-T) for 1 hour at RT. Following three successive washes (10, 10, 15 minutes) in TBS-T, the membranes were incubated

Table 2.1: Solution recipes for SDS-PAGE/Western blot

Gels or Buffers	Recipes	mL(g)
15% Resolving Gel (20mL)	30% acrylamide	10 mL
	1.5M Tris pH 8.8	5 mL
	10% SDS	0.2 mL
	10% AP	0.2 mL
	TEMED	0.008 mL
	ddH2O	4.6 mL
5% Stacking Gel (10mL)	30% acrylamide	1.7 mL
	1M Tris pH 6.8	1.25 mL
	10% SDS	0.1 mL
	10% AP	0.1 mL
	TEMED	0.01 mL
	ddH2O	6.8 mL
1X Running Buffer (1000mL)	Tris-Base pH 8.3	3.02 g
	Glycine	14.42 g
	SDS	1 g
	ddH2O	to 1000 mL
1X Transfer Buffer (1000mL)	Tris-Base pH 8.3	3.02 g
	Glycine	14.42 g
	Methanol	200 mL
	ddH2O	to 1000 mL
1X TBS (1000mL)	Tris-Base pH 7.5	3.02 g
	NaCl	4.39 g
	ddH2O	to 1000 mL
1X TBS-T (1000mL)	Tris-Base pH 7.5	3.02 g
	NaCl	4.39 g
	Tween-20	0.5 mL
	ddH2O	to 1000 mL

with ECL or ECL plus reagent and developed on Kodak BioMax MR Film. Then, the films were scanned and process with Image-J software (NIH) for quantification. Finally, each membrane was re-probed for tubulin, as a loading control, in order to correct for protein loading differences. To get rid of the membrane-associated primary and secondary antibodies in order to re-probe the membrane for other proteins, the membrane was incubated in freshly made stripping buffer (25mM glycine pH 2.0, 1% SDS) at room temperature for 20 minutes with gentle agitation. Following this, the membrane was washed with TBS-T three times for 10 minutes, it was blocked again in 5% non-fat milk for 2 hours at room temperature, and then immunoblotted with antibodies as described above. After ECL detection, the membranes were stored at 4°C.

2.7. ASO Transfection

ASO transfection was carried out in 60mm dishes (Corning) with the aid of lipofectin reagent according to the manufacturer's instruction. The day before transfection, 3 ml of TCCB cells at ~1.6 X 10^5/ml were seeded into each dish and equilibrated overnight in their regular growth medium without antibiotics (see details in section 2.2). This generally resulted in a cell confluency of about 40% on the next day. Then, depending on the protein(s) proposed to downregulate, 20µl of Bcl-2 ASO (100µM) or/and 20µl of Clusterin ASO (100µM) was mixed gently with 350-330µl of Opti-MEM in each eppendorf tube to the final volume of 370µl. As well, 7µl of lipofectin reagent was diluted into 23µl of Opti-MEM in a second set of eppendorf tubes and incubated at room temperature for 30 minutes. Then, the ASO/Opti-MEM mixture was combined with the lipofectin/Opti-MEM mixture in one set of tubes (400µl/tube) and incubated at room temperature for 20 minutes to allow the ASO-lipofectin complexes to form. In the

meantime, the TCCB cell layer was washed once with 4ml of growth medium without FBS or antibiotics. This was followed by another wash with Opti-MEM serum-reduced medium and replenishment with 1.6ml of Opti-MEM. Then, the 400µl of the ASO-lipofectin complexes were added into each dish, and mixed thoroughly with the pre-existing Opti-MEM medium. Thus, the final ASO concentration was 1000nM in each dish. Following 4 hours of incubation at 37°C and 5% CO_2, the ASO-lipofectin transfection mixture was replaced with 3ml of regular growth media without antibiotics. The cells were then cultured at the regular condition (37°C, 5% CO_2) for 24 hours prior to the combined TRAIL treatments or protein extraction/SDS-PAGE/Western blots. Based on the proposed studies, the successive ASO transfection(s) at the second and the third days were also applied prior to the further protein extractions (see detailed experimental procedures in chapter 3). In the meantime, the mismatched (MM) oligodeoxynucleotides were also transfected into the cells as a transfection control and non-transfected cells were used as a negative control in each experimental condition.

2.8. Live/Dead Assay

Live/Dead assays were conducted on both co-culture multicellular spheroids (MCS) following TRAIL treatment and TCCB cells after TRAIL-ASO combination treatments.

The co-culture spheroids were grown on 1% agarose in DMEM medium supplemented with 10% FBS by using the liquid overlay culture technique [215]. Briefly, human TCCB cells (MGH-U3) and normal fibroblast cells (F2P6) were seeded in a 96-well plate at 3000 tumor cells and 10,000 fibroblast cells per well onto a 50 µl semi-solid agarose layer, and maintained in 200 µl of DMEM medium supplemented with 10% FBS.

Small MCS of each kind were combined after 3 days by pipette transfer. TCCB cell (MGH-U3) spheroid will completely engulf the fibroblast (F2P6) spheroid providing a three-dimensional *in vitro* TCCB model to study topical drug application. The culture media was changed daily to provide optimal cell growth. The generated co-cultured (MGH-U3/F2P6) spheroids were then treated with 300ng/ml of TRAIL in culture medium. After initial two hours, cells were treated with fresh solution of drug as the initial dose would have been diluted by infusion into the agarose. Total dwell-time was 6 hours. TRAIL containing media was then removed and replaced with regular culture media, and culturing continued to the appropriate time-points. For the detection of survival, spheroids were treated with 5 μM SYTO-16 live cell nucleic stain and 5 μM dead cell stain propidium iodide for 1 hour at 37°C.

For the live/dead assays after TRAIL-ASO combination treatment, the proposed TCCB cells were seeded into a 24 well plate at 30,000/well and equilibrated overnight in the growth medium without antibiotics under standard conditions (5% CO2, 37°C). Then, the cells were transfected with Bcl-2 ASO and/or Clusterin ASO as previously described in section 2.7, using the non-transfected and MM-transfected cells as controls. Twenty-four hours after transfection, the TCCB cells were treated with recombinant human TRAIL at 300ng/ml for 4 hours and maintained in renewed growth medium without antibiotics for another 24 hours. Cells were then exposed to 7.5 μM SYTO-16 live cell nucleic stain and 2.5 μM dead cell stain propidium iodide for 1 hour at 37°C to detect the cellular response.

The stained plated cells were then placed on a Zeiss Axioplan upright digital imaging microscope equipped with a cooled CCD camera (Sensys Cooke).The cells were

focused upon with a plan-neofluar 10X lens. Using Metamorph software (Universal Imaging Corporation, Downingtown, PA, USA), cell images were recorded using both fluorescein isothiocyanate (FITC) and Cy3 filters.

2.9. Statistical Analysis

Experiments were repeated at least three times under each condition. The resultant data under different experimental conditions were represented by the average ± S.E. (Standard Error), using MS Excel software. Statistical significance was determined using student's t-test, $p<0.05$. The Student-Newman-Keuls (SNK) multiple comparisons test was used to determine statistical differences among more than two groups.

Chapter 3: Results

3.1. Effect of TRAIL on TCCB Cells

3.1.1. Sensitivity of TCCB Cells to TRAIL

With the advance in understanding TRAIL-induced apoptosis in many other malignant cells, the research examining TRAIL as a potential anti-TCCB reagent has been carried on in this study. Eleven human TCCB cell lines were obtained and analyzed to investigate the sensitivity of TCCB cells to TRAIL. For each cell line, the cells were treated by recombinant human TRAIL with three–fold serial dilutions starting from 1000ng/ml (Figure 3.1). After 24 hours of TRAIL incubation, MTT assays were carried out to determine the percentage of cell death using the standard formula [(1 - optical density of cells treated / optical density of untreated cells) x 100]. As shown in Figure 3.1, TCCB cells showed a differential response to TRAIL. According to the percentage of cell death at 1000ng/ml of TRAIL, TCCB cells can be divided into three subgroups: TRAIL-sensitive cells (>65% of cell death) including RT-4, UMUC14, HTB-9 (5637), MGH-U3 and UMUC3 cell lines; TRAIL-partially resistant cells (>10% of cell death, but < 45%) which consist of RT-112, 253J, UMUC9, T24 and UMUC6 cell lines; and TRAIL-resistant cells (< 3%), CRL-1472 (HT-1376).

Further in both TRAIL-sensitive and -partially resistant cells (Figure 3.1 A and B), the percentage of cell death seems to correlate with TRAIL concentration (0 -100ng/ml). The higher concentration of TRAIL applied to the cells, the greater percentage of cell death was observed. However, in the TRAIL-sensitive TCCB cells, a plateau was reached at a TRAIL concentration greater than 100ng/ml (Figure 3.1 A). This asymptotic response was not observed in the TRAIL-partially resistant TCCB cells (Figure 3.1 B). Conversely, as shown in Figure 3.1 C, the bladder cancer cell line CRL-1472 (HT-1376)

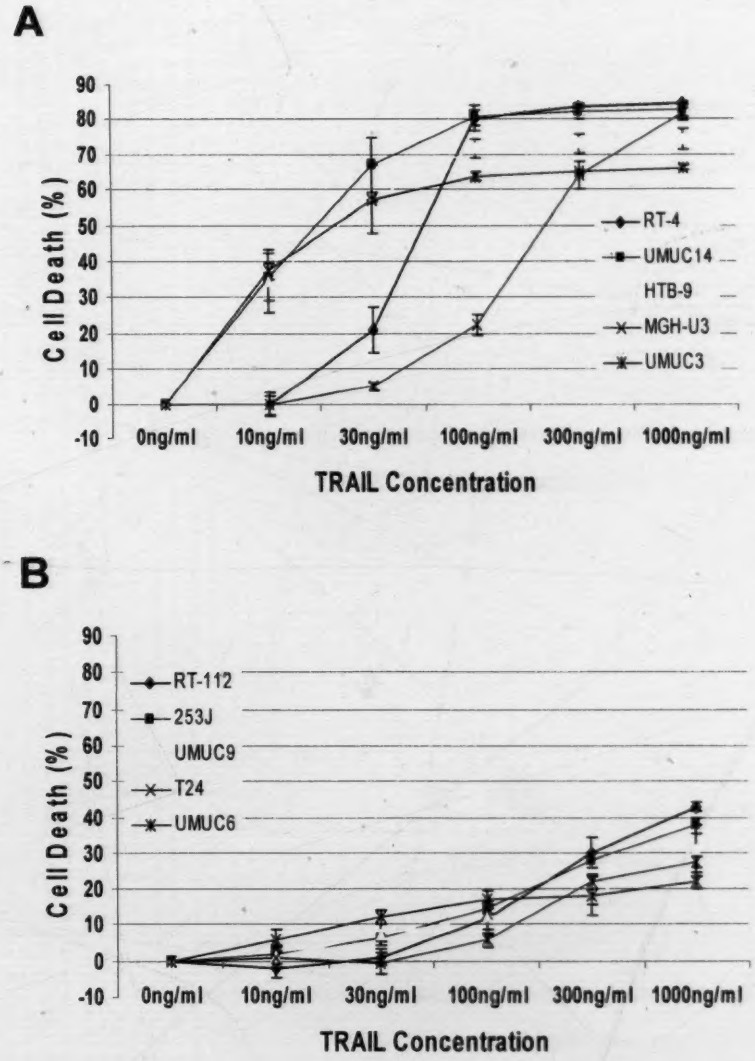


Figure 3.1: Sensitivity of TCCB cell lines to TRAIL-induced cell death.

C

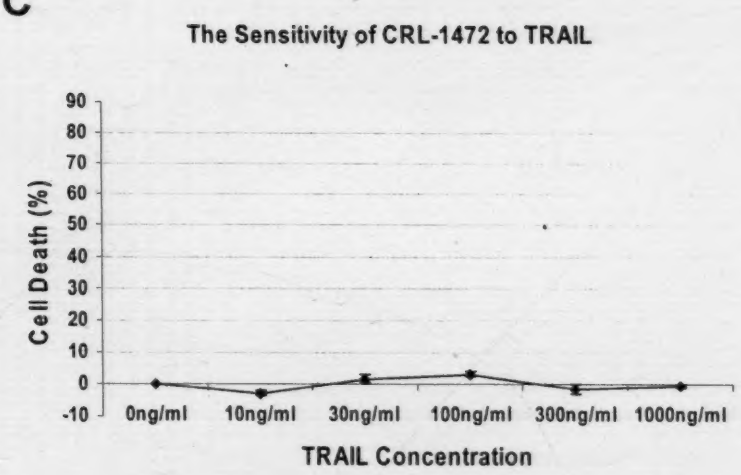


Figure 3.1 Continued: To determine the sensitivity of TCCB cell lines to recombinant human TRAIL at various concentrations (dose dependency), MTT assays were carried out on RT-4, UMUC14, HTB-9, MGH-U3, UMUC3, RT-112, 253J, UMUC9, T24, UMUC6, and CRL-1472 cell lines after 24 hours of TRAIL treatment. Based on the percentage of cell death at 1000ng/ml of TRAIL, (A) RT-4, UMUC14, HTB-9, MGH-U3 and UMUC3 cell lines are sensitive to TRAIL-induced cell death, (B) RT-112, 253J, UMUC9, T24, and UMUC6 cell lines are partially resistant to TRAIL, while (C) CRL-1472 showed total resistance to TRAIL-induced cell death. The data represent the average percentage of cell death ± S.E. (standard error) from at least three independent experiments.

exhibited total resistance to TRAIL regardless of the applied concentration of TRAIL, despite having surface expression of death receptors (DR4 and DR5) [prior work in Moore lab by Loretta Vallee-Lucic]. To further understand the differential sensitivity of TCCB cells to TRAIL, we have investigated the TRAIL-induced apoptotic signals in these cell lines.

3.1.2. Time-dependent Response of TCCB Cells to TRAIL

As previously described in section 1.3.2, subsequent to the binding of TRAIL to the death receptors, TRAIL may induce apoptosis by activating the apoptotic signals through the extrinsic pathway and tBid-mediated intrinsic pathway. To further examine the apoptotic signals in TCCB cells after TRAIL treatment, each cell line was treated with 300ng/ml of recombinant human TRAIL for escalated time periods (untreated, 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, 12 hours, and 24 hours). Subsequently, the activation of both death receptor pathway (caspase-8 → caspase-3 → DFF45) and mitochondrial pathway (caspase-9 → caspase-3 → DFF45), as well as the initiation and declination of these apoptotic signals, were successfully detected in the TRAIL responsive TCCB cells through SDS-PAGE/Western blots (Figure 3.2). Although the signaling dynamics differed slightly in the timing among these TCCB cell lines, they all yielded the similar information but to varying degrees. As shown in Figure 3.2 A, B and C, the initiation of death receptor pathway is evident by the cleavage of caspase-8 (cleaved caspase-8, 18kDa). The mitochondrial pathway is marked by the activation of caspase-9 (cleaved caspase-9, 35kDa). The cross-talking between these two pathways is through the cleaved Bid (tBid, 15kDa). DNA fragmentation factor 45 (DFF45/ICAD) was cleaved by active caspase-3 (cleaved caspase 3, 17kDa) during

apoptosis, which causes the activation of the caspase-activated DNase (CAD/DFF40) and then DNA degradation. In addition, tubulin (55kDa) was detected as a loading control, showing that proteins were equally loaded in each experiment.

As a representative example shown here in Figure 3.2 A, after TRAIL exposure for 2 hours, the activation of caspase-8, Bid, caspase-9 and caspase-3, and the cleavage of DFF45 (ICAD) were found in TRAIL-sensitive TCCB cells, HTB-9 (5637). Those apoptotic signals reached to the maximal levels at the 3 hour time point and significantly decreased after 6 hours of TRAIL treatment. No apoptotic signaling was detected after 12 hours of TRAIL exposure. Similar results were also observed in other TCCB cell lines, for example, MGH-U3 cells (Figure 3.2 B), where both of the extrinsic and intrinsic apoptotic signals were initiated after 2 hours of exposure to TRAIL, but lingered longer than 12 hours with a slow activation of final executioners, especially the cleavage of DFF45. In the most TRAIL-sensitive TCCB cells, UMUC14, the apoptotic signaling transduction started earlier than other TRAIL-sensitive TCCB cell lines, as early as 1 hour following TRAIL exposure, and diminished after 12 hours (Figure 3.2 C). However, on the contrary to these TRAIL-sensitive TCCB cells, no activated death signal in either the death receptor pathway or mitochondrial pathway was detected in the TRAIL-resistant TCCB cell line, CRL-1472 (Figure 3.2 D), although the death receptors (DR4 and DR5) were detected on the cell surface.

Taken together, these results suggest that TRAIL-induced apoptotic signaling is decisive for the sensitivity of TCCB cells to TRAIL, which may occur as a consequence of both the death receptor pathway and tBid-mediated mitochondrial pathway. The

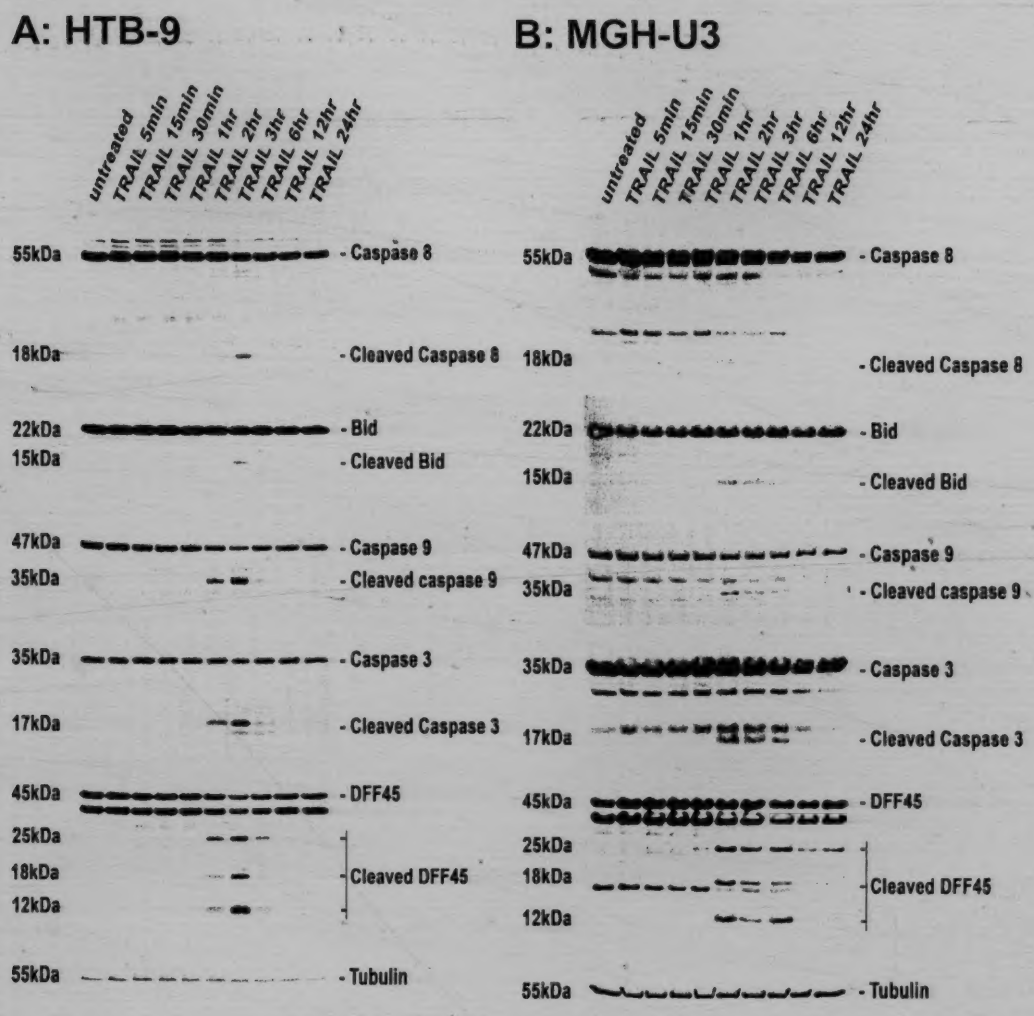


Figure 3.2: TRAIL-induced apoptotic signaling in TCCB cells. TCCB cell lines were treated with 300ng/ml of recombinant human TRAIL for escalated time periods. The initiation of death receptor and mitochondrial pathway is evident by the cleaved caspase-8 (18kDa) and the cleaved caspase-9 (35kDa) respectively. The cross-talking between these two pathways are mediated by cleaved Bid (tBid, 15kDa). As the result, the activated caspase-3 cleaves DFF45/ICAD, resulting in apoptotic cell death. (A): HTB-9; (B) MGH-U3; (C): UMUC14.

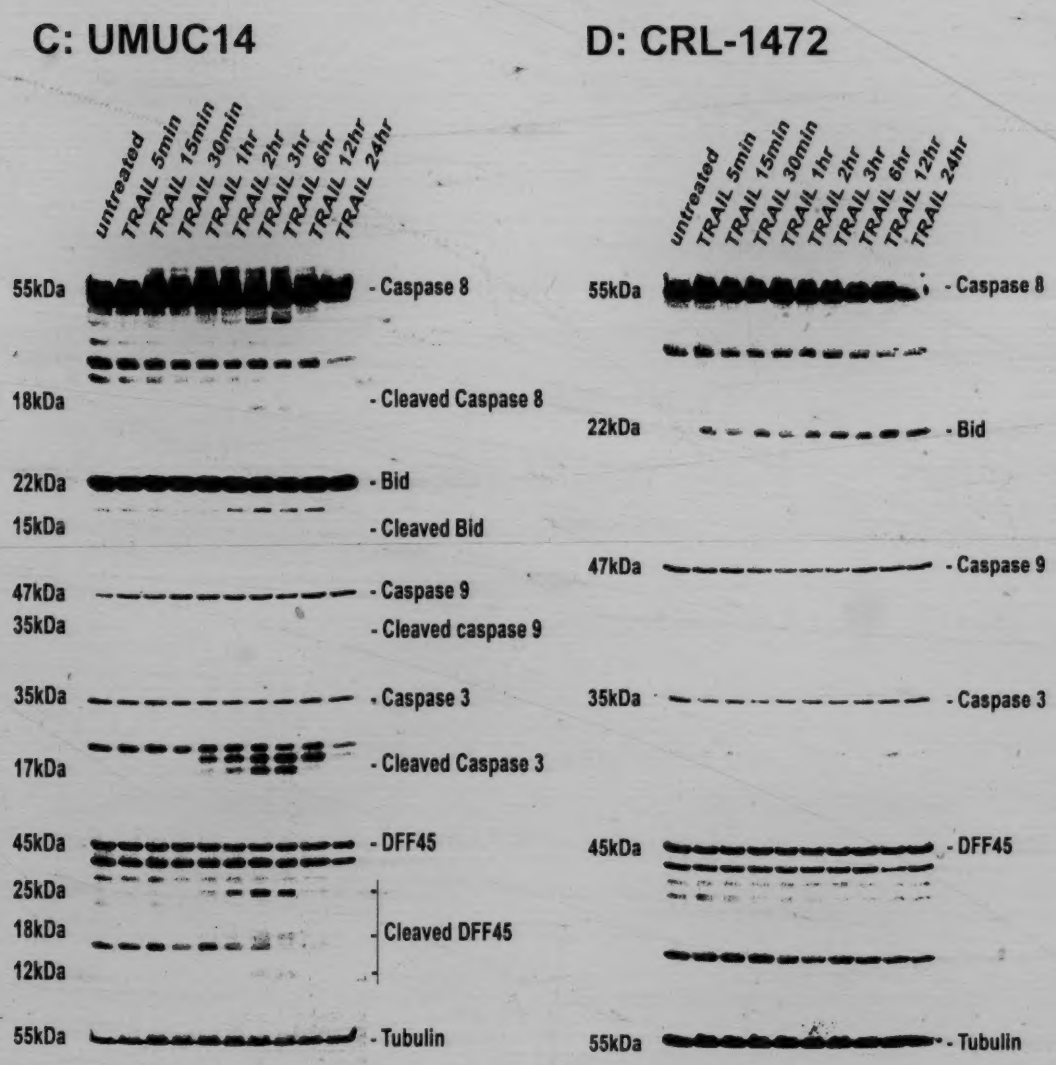
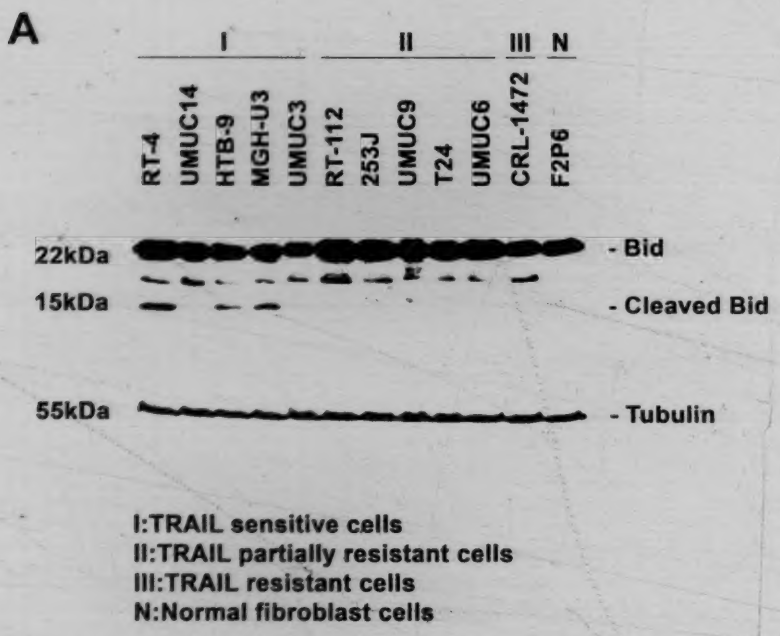


Figure 3.2 continued: As a comparison, after TRAIL treatment, no apoptotic signal transduction was found in CRL-1472 (HT-1376), the TRAIL-resistant TCCB cells (D).

increase or decrease in the activation of apoptotic signaling on death receptor pathway and/or mitochondrial pathway may modify the TRAIL sensitivity of TCCB cells.

3.1.3. Relationship of Bid Cleavage to TRAIL Sensitivity in TCCB Cells

Bid, as previously described in section 1.3.2.3, is a pro-apoptotic member of Bcl-2 family and is the cross-talking link between the death receptor pathway and mitochondrial pathway in TRAIL-induced apoptosis. Following the binding of TRAIL to the death receptors, activated caspase-8 causes the cleavage of Bid (p22 → p15) to initiate the intrinsic apoptotic pathway by inducing cytochrome *c* release from mitochondria and the subsequent activation of caspase-9. To further examine the relationship of Bid cleavage with the TRAIL sensitivity of TCCB cells, 11 TCCB cell lines and 1 normal fibroblast cell line were seeded simultaneously and treated with 300ng/ml of recombinant human TRAIL for 4 hours. Whole cell proteins were then isolated after TRAIL treatment. After protein assay, equal amounts of protein were loaded and separated through SDS-PAGE. The expression of cleaved or truncated Bid (tBid) was determined by immunoblotting (Figure 3.3 A). The experiments were repeated three times, and the relative levels of tBid in these cell lines were quantified and graphed using tubulin as a loading control. As shown in Figure 3.3 B, the amount of tBid triggered by TRAIL/DR binding appears to be correlated with the sensitivity of TCCB cells to TRAIL, especially in the TRAIL-partially resistant cells. The exceptions are the two TRAIL-sensitive cell lines: UMUC14 and UMUC3 (Figure 3.3 A and B). However, by comparing the total Bid expression levels in untreated cells with the activated tBid following TRAIL treatment in each TCCB cell line, the reason that UMUC14 and UMUC3 have low level



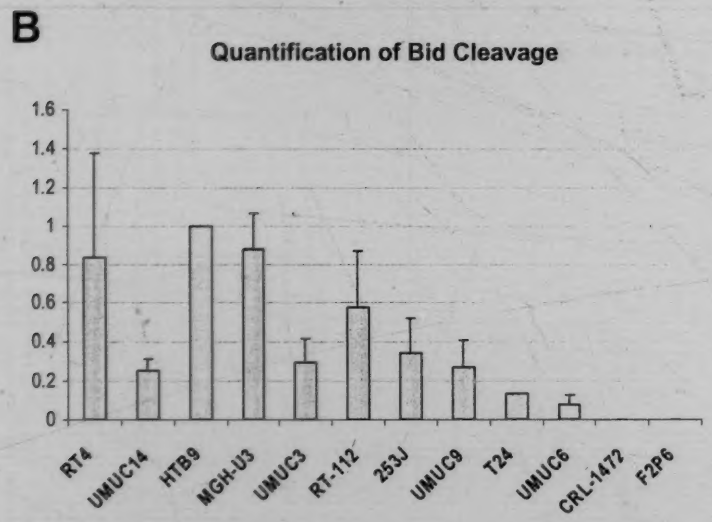


Figure 3.3: Examination of TRAIL-induced Bid cleavage in TCCB cells. (A) The cleaved Bid was examined by SDS-PAGE/Western Blots in TCCB cell lines and normal fibroblast F2P6 following 4 hours of TRAIL (300ng/ml) treatment. (B) Quantitative analysis of Bid cleavage level after normalization to tubulin level. The data represent the average ± S.E. (standard error) from three independent experiments.

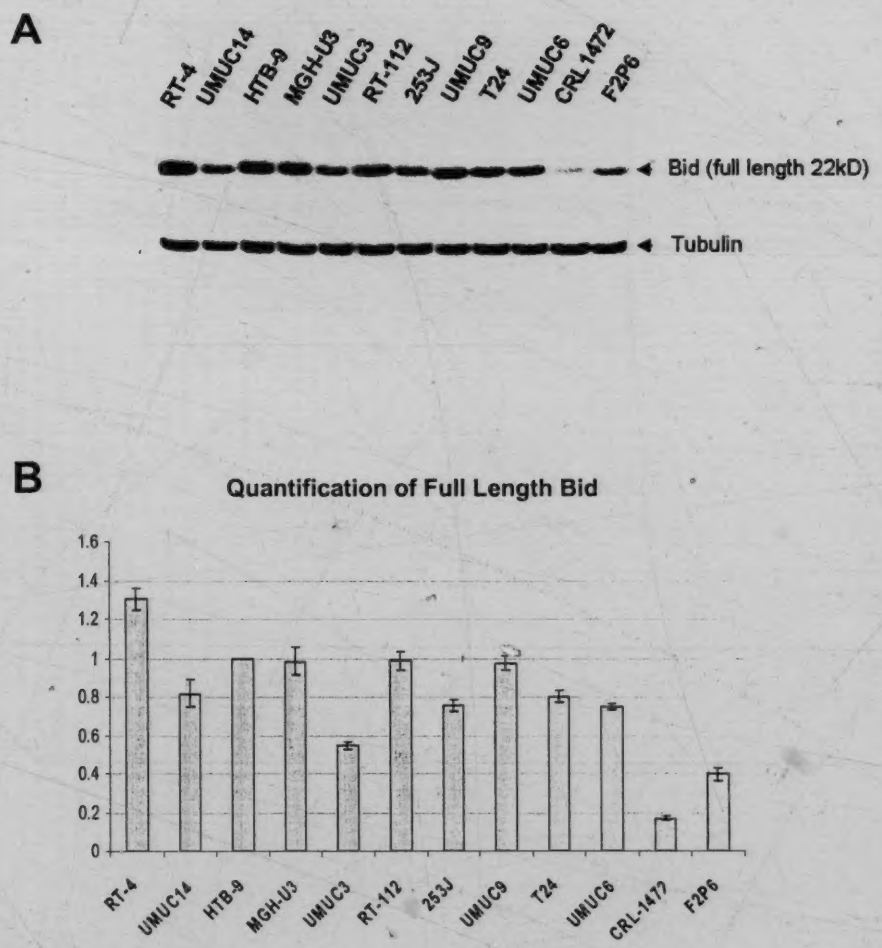


Figure 3.4: Cellular expression of full length Bid in TCCB cells. (A) The full length Bid expression levels were examined through SDS-PAGE/Western blots in untreated TCCB cells and normal fibroblast F2B6 cells, using tubulin as a loading control. (B) Quantitative analysis of the relative levels of full length Bid in these cell lines. From left to right, it is the most TRAIL-sensitive TCCB cell line to TRAIL-resistant cell line. The data represent the average ± S.E. (standard error) from three independent experiments.

of Bid cleavage seems to be related to their low expression of full length Bid (Figure 3.4). Thus, in the TRAIL-partially resistant TCCB cells, TRAIL sensitivity seems to correlate with how much apoptotic signals can be transferred or directed to mitochondrial pathway. These results suggest that an approach to further enhance the intrinsic apoptotic signaling transduction by downregulation of the anti-apoptotic proteins on mitochondrial pathway may sensitize the TRAIL-partially resistant TCCB cells to TRAIL-induced apoptotic cell death.

3.1.4. Safety of Recombinant Human TRAIL

To examine the safety of using TRAIL as an anti-cancer agent, normal fibroblast cell line F2P6 was treated by recombinant human TRAIL with three-fold serial dilution starting from 1000ng/ml for 24 hours as previously conducted on TCCB cells. Then, MTT assay was carried out to determine the percentage of cell death. As shown in Figure 3.5 A, the normal fibroblast F2P6 cells showed no response to TRAIL, which is consistent with the results of apoptotic signaling detection through Western blots (Figure 3.5 B). The normal fibroblast cells were treated with 300ng/ml of recombinant human TRAIL for escalated time up to 24 hours. No active caspase-8, -3, -9, cleaved Bid or DFF 45 was detected in the TRAIL-treated normal fibroblast F2P6 cells.

Further, the selective cytotoxicity of TRAIL was examined on our co-cultured *in vitro* model of TCCB (Figure 3.6). The co-culture spheroids of human TCCB cells MGH-U3 and normal fibroblast cells F2P6 were treated with recombinant human TRAIL at 300ng/ml in culture medium. After initial two hours, cells were treated with fresh solution of drug since the initial dose would have been diluted by infusion and redistribution into the agarose. Total dwell-time was 6 hours; after which the drug was

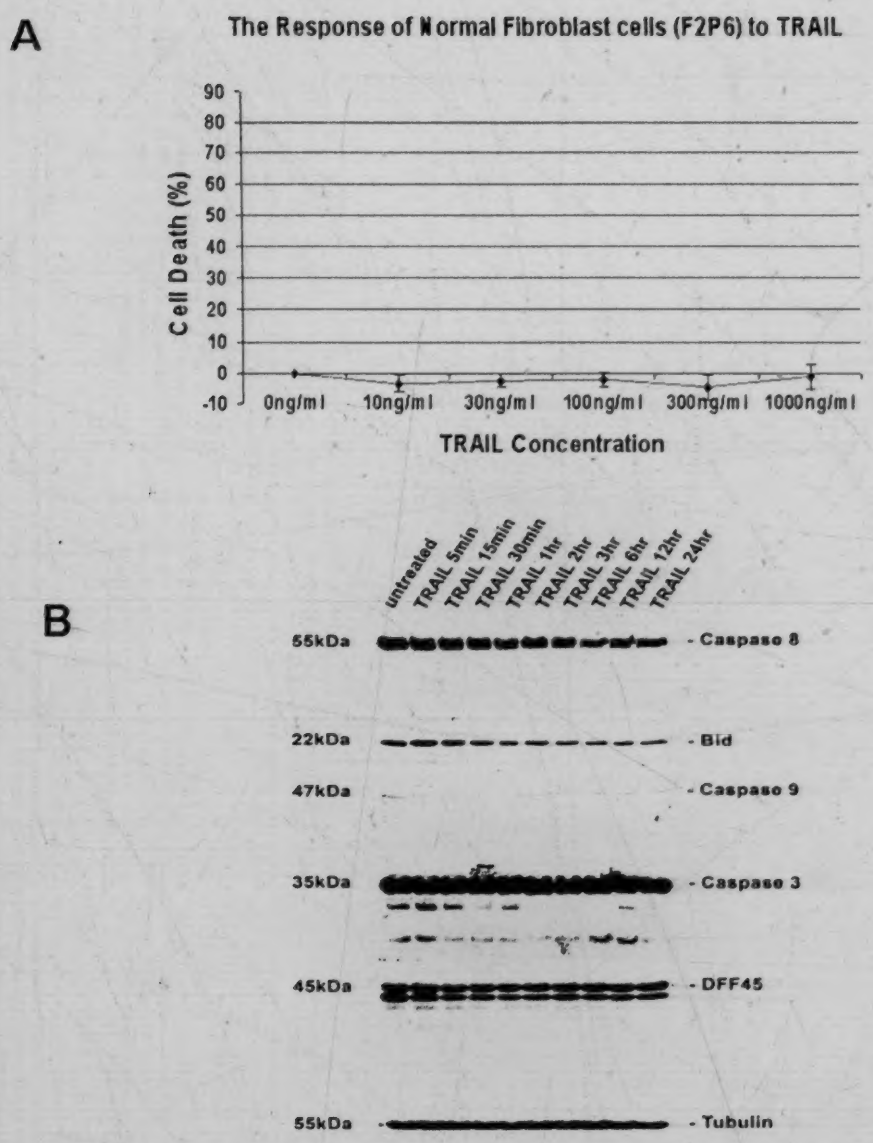


Figure 3.5: Response of normal fibroblast F2P6 cells to TRAIL. (A) No TRAIL-induced cell death was observed in normal fibroblast F2P6 cells. (B) Following TRAIL treatment for escalated time periods, no apoptotic signaling was observed in either death receptor pathway or mitochondrial pathway in fibroblast F2P6 cells.

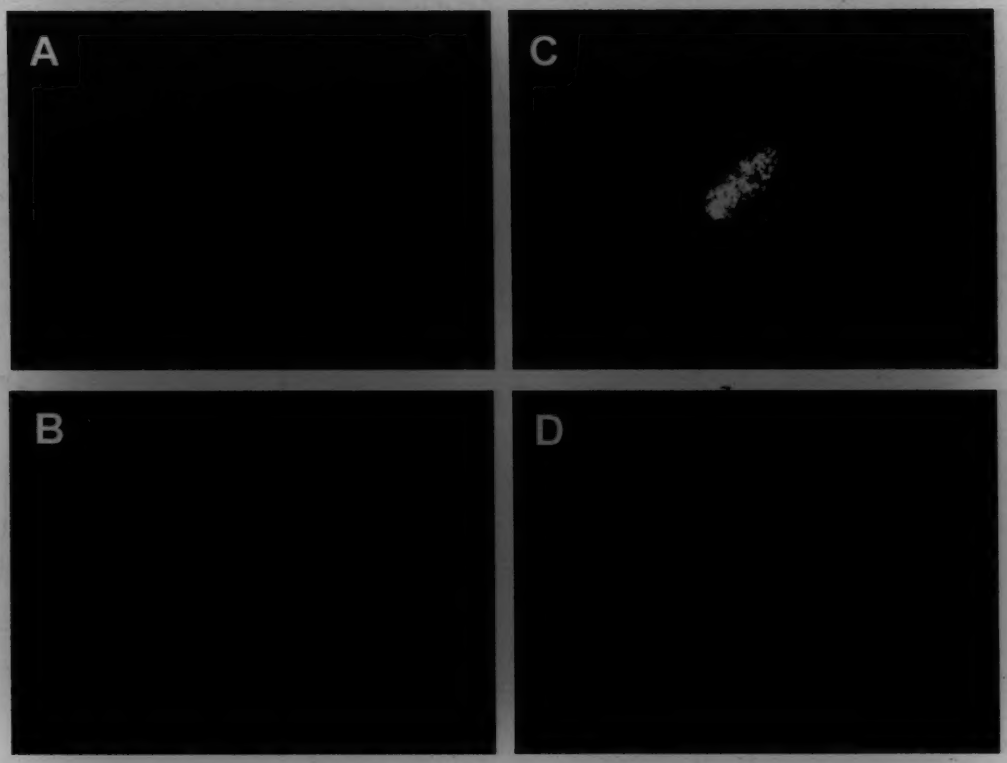


Figure 3.6: TRAIL selective cytotoxicity of TCCB cells in co-culture model. The live/dead assays were conducted on our co-cultured *in vitro* model of TCCB. The co-culture spheroids of human TCCB cells MGH-U3 (peripheral) and normal fibroblast cells F2P6 (central) were generated as previously described in section 2.8. The SYTO 16 fluorescence was identified under a FITC filter, while the Propidium Iodide signal was identified under a Cy3 filter indicating dead cells. (A) The non-TRAIL treated co-culture spheroid was used as a control. (B) Normal fibroblast F2P6 spheroid treated with TRAIL at 300ng/ml (6hrs dwell-time). The image was recorded 3 days after TRAIL withdrawal. (C) Co-cultured spheroid treated with TRAIL at 300ng/ml (6hrs dwell-time). The image was recorded 1 day after TRAIL withdrawal. (D) Co-cultured spheroid treated with TRAIL at 300ng/ml (6hrs dwell-time). The image was recorded 3 days after TRAIL withdrawal.

removed and substituted with regular medium and culturing continued to the appropriate time-points. Then spheroids were stained for 1 hour with 5 μM of SYTO 16 live cell nucleic stain and 5 μM of dead cell stain Propidium Iodide at 37°C.

Images were analyzed through a Zeiss Axioplan upright digital imaging microscope. As indicated in a previous publication from Moore laboratory, the immunohistochemical study in co-culture spheroids demonstrated a central localization of fibroblast cells and a peripheral orientation of bladder cancer cells [215]. The results showed no response of normal fibroblast spheroids (F2P6) to TRAIL in Figure 3.6 B, which is consistent with the results detected though MTT assay and Western Blots. As shown in Figure 3.6 C and D, the topical application of TRAIL in our co-cultured MGH-U3/F2P6 spheroids showed selective killing of peripheral TCCB cells MGH-U3 with sparing of the central normal cells F2P6, which gives the further support to the safety of TRAIL in potential clinical application.

3.2. Effect of ASO on TCCB Cells

3.2.1. Expression of Bcl-2 and Clusterin in TCCB Cells

The anti-apoptotic function of Bcl-2 and Clusterin, as indicated in section 1.3.3, has been demonstrated in many malignancies. To examine if the expression levels of Bcl-2 and Clusterin are correlated with the TRAIL sensitivity of TCCB cells, the cells from each cell line were seeded simultaneously and equilibrated overnight to allow the cell attachment. Then, whole cell proteins were isolated and equal amounts of proteins were separated by SDS-PAGE. The expression of Bcl-2 and Clusterin were detected with western blotting (Figure 3.7 A). Quantitative analysis of the relative expression level of Bcl-2 and Clusterin is presented as the mean of triplicate analysis after normalization to tubulin level (Figure 3.7 B, C). The cell lines were ordered from the most TRAIL-sensitive cells to the most TRAIL-resistant cells in the Figure 3.7. Although no close correlation of TRAIL sensitivity to the protein expression level of Bcl-2 and Clusterin can be detected in the examined TCCB cells, the modulation of their protein expression in the cells may affect the sensitivity to TRAIL as previously described in the section 1.3.3.

3.2.2. Downregulation of Bcl-2 and Clusterin Protein Expression through ASO Transfection in TRAIL-partially Resistant TCCB Cells

According to the Gleave group's published results, ASO treatment (100, 500, 1000mM) on the bladder cancer cells can significantly reduce mRNA level by 22, 59, 95% respectively [209]. To determine if Bcl-2 phosphorothioate ASO and Clusterin phosphorothioate ASO can downregulate the corresponding protein level in TCCB cells,

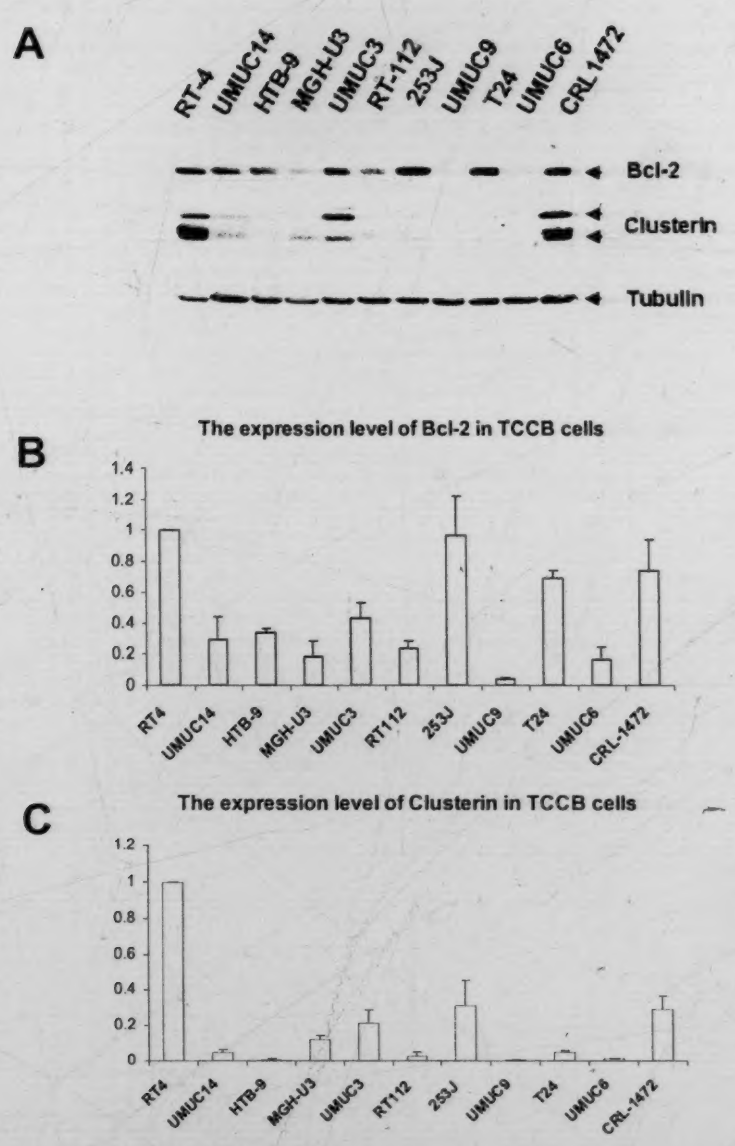


Figure 3.7: Relative protein expression of Bcl-2 and Clusterin among untreated TCCB cell lines. (A) TCCB cell lines were seeded overnight and the whole cell proteins were isolated at the same time. SDS-PAGE/Western Blot was carried out to determine the expression of Bcl-2 and Clusterin. Quantitative analysis of relative expression level of Bcl-2 and Clusterin is presented as the mean of triplicate analysis after normalization to tubulin level (B, C). Cells were ordered (left to right) from the most TRAIL-sensitive to the most TRAIL-resistant.

the ASO transfection experiments were carried out on TRAIL-partially resistant cell lines, T24 and RT-112. In each experiment, TCCB cells were seeded in the dishes which were divided into three groups (group-1, -2, -3). After the cells were equilibrated overnight, the cells were transfected with Bcl-2 phosphorothioate ASO or Clusterin phosphorothioate ASO at 1000nM for 4 hours per day for one day for group-1, two days for group-2, and three days for group-3 respectively, using mismatch (MM) oligodeoxynucleotides as a transfection control. Whole cell proteins were then isolated 24 hours after the final ASO treatment under each experimental condition. Western Blots were carried out to determine the changes in protein expression levels of Bcl-2 and Clusterin (Figure 3.8 A - 3.11 A). The quantitative analyses of Bcl-2 and Clusterin level were subsequently performed by normalization to the level of tubulin (Figure 3.8 B- 3.11 B).

As shown in Figure 3.8, the expression level of Bcl-2 protein in the TRAIL-partially resistant TCCB cells T24 was significantly reduced about 50% after 4 hours of Bcl-2 ASO treatment (one time, group-1). The downregulation of Bcl-2 protein expression level appeared to be similar (~50%) even after the cells were transfected twice or three times with Bcl-2 ASO (group-2 and -3). In addition, a decrease in the Clusterin protein expression was achieved through Clusterin ASO transfection. The downregulation level of Clusterin appeared to reach similar levels (~60%) in T24 cells in the three groups whether they were transfected with Clusterin ASO once, twice, or three times (Figure 3.9). The treatments of Bcl-2 ASO and Clusterin ASO also successfully decreased the corresponding protein expression in the other TRAIL-partially resistant TCCB cell line RT-112 and revealed similar results as shown in Figure 3.10 and 3.11.

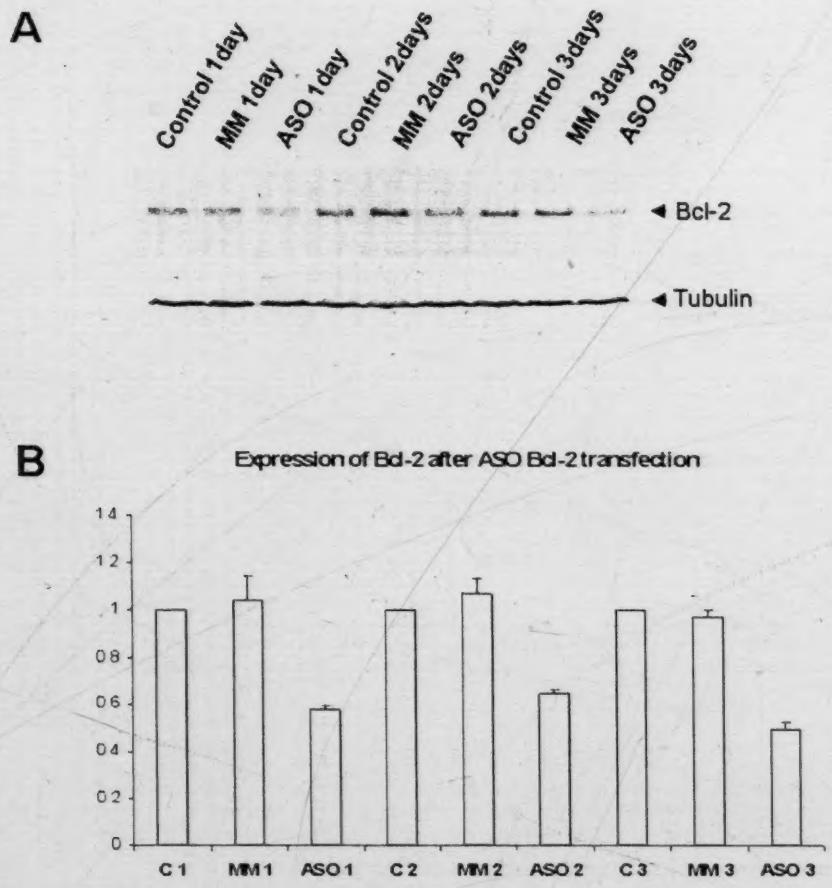


Figure 3.8: Downregulation of Bcl-2 protein expression through Bcl-2 ASO transfection on T24. (A) T24 cells were seeded and transfected with 1000nM of Bcl-2 phosphorothioate ASO for 4 hours per day for 1 day, 2 days and 3 days with mismatch (MM) oligodeoxynucleotides as a transfection control. Whole cell proteins were isolated 24 hours after ASO treatments. SDS-PAGE/Western Blots were carried out to determine the protein expression of Bcl-2. (B) The quantitative analysis of Bcl-2 expression level is presented as the average of three times detection with standard error after normalization to tubulin level.

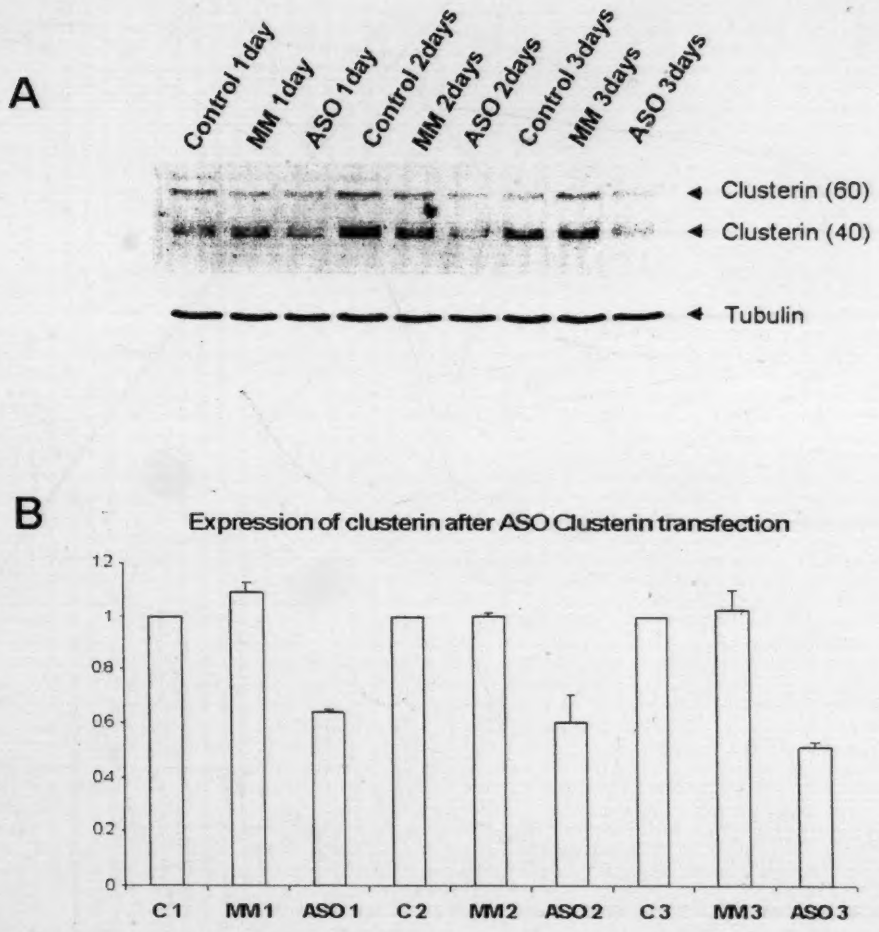


Figure 3.9: Downregulation of Clusterin protein expression through Clusterin ASO transfection on T24. (A) T24 cells were seeded and cultured overnight. Then the cells were transfected with 1000nM of Clusterin ASO for 4 hours per day for 1 day, 2 days and 3 days. Mismatch (MM) oligodeoxynucleotides were used as a transfection control. Proteins were isolated 24 hours after ASO treatments. SDS-PAGE/Western Blots were performed to determine the protein expression of Clusterin. (B) The quantitative analysis of Clusterin level is presented after being normalized to tubulin level.

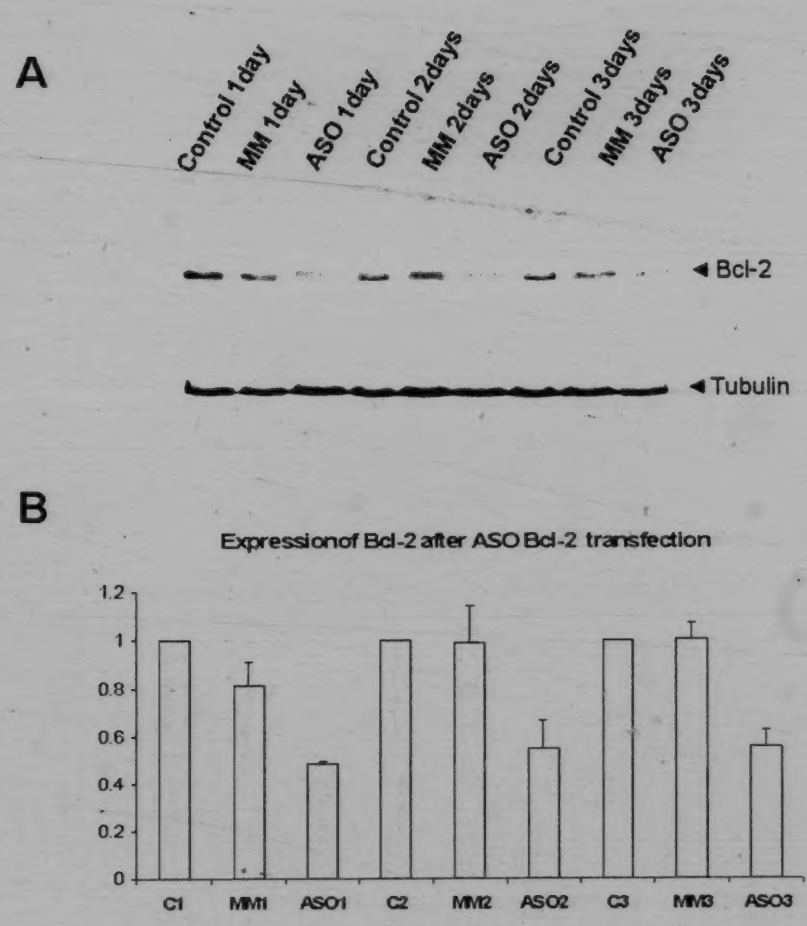


Figure 3.10: Downregulation of Bcl-2 protein expression through Bcl-2 ASO transfection on RT-112. (A) RT-112 cells were seeded and cultured overnight to allow for cell attachment. Cells were then transfected with Bcl-2 phosphorothioate ASO at 1000nM for 4 hours per day for 1 day, 2 days and 3 days, with mismatch (MM) oligodeoxynucleotides as a transfection control. Proteins were isolated 24 hours after ASO treatments. Western Blots were carried out to determine the protein expression of Bcl-2. (B) The quantitative analysis of Bcl-2 level is shown after normalization to tubulin level.

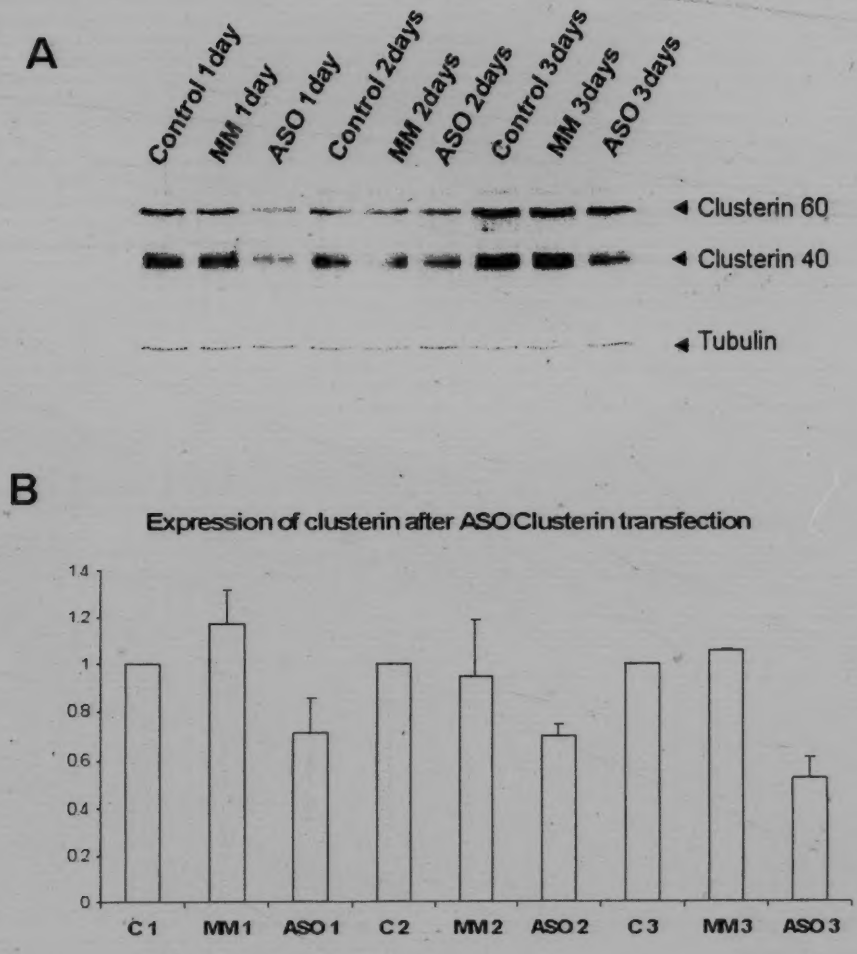


Figure 3.11: Downregulation of Clusterin protein expression through Clusterin ASO transfection on RT-112. (A) RT-112 cells were seeded and cultured overnight. Then the cells were transfected with 1000nM of Clusterin phosphorothioate ASO for 4 hours per day for 1 day, 2 days and 3 days with mismatch (MM) oligodeoxynucleotides as a transfection control. Proteins were isolated 24 hours after ASO treatments. Western Blots were performed to determine the protein expression of Clusterin. (B) The quantitative analysis of Clusterin is shown after normalization to tubulin.

Taken together, these results provided the basis for designing further ASO combined TRAIL treatment on those TRAIL-partially resistant cell lines in section 3.3.

3.2.3. Bcl-2 and Clusterin Protein Expression in TRAIL-partially Resistant TCCB Cells after Bcl-2 ASO and Clusterin ASO Combined Transfection

As demonstrated above, the treatment of Bcl-2 ASO and Clusterin ASO can successfully downregulate the corresponding protein expression levels in TRAIL-partially resistant TCCB cells 24 hours after ASO transfection. To examine if Bcl-2 ASO and Clusterin ASO combined transfection could synergize or interfere with their function on decreasing protein expression, TRAIL-partially resistant cells T24, RT-112 and 253J were explored. Cells were transfected with Bcl-2 phosphorothioate ASO and/or Clusterin phosphorothioate ASO at 1000nM for 4 hours with mismatch (MM) phosphorothioate oligodeoxynucleotides as a transfection control. Whole cell proteins were isolated 24 hours after ASO transfection. Western Blots were carried out to determine the expression levels of Bcl-2 and Clusterin (Figure 3.12-3.14 A and C). The quantitative analysis of Bcl-2 and Clusterin after normalization to tubulin level is presented as the mean of three individual experiments with standard error (Figure 3.12-3.14 B and D).

As shown in the Figure 3.12 A and B, the treatment of Bcl-2 ASO successfully reduced 50% of the Bcl-2 protein expression 24 hours after 4-hour ASO transfection in T24 cells, and Clusterin ASO transfection did not affect the protein expression of Bcl-2. The transfection of Bcl-2 ASO plus Clusterin ASO can downregulate the expression level of Bcl-2 to 65% compared with untreated cells, but not as significantly as the transfection of Bcl-2 ASO alone (the fifth column in Figure 3.12 B). The same results were also

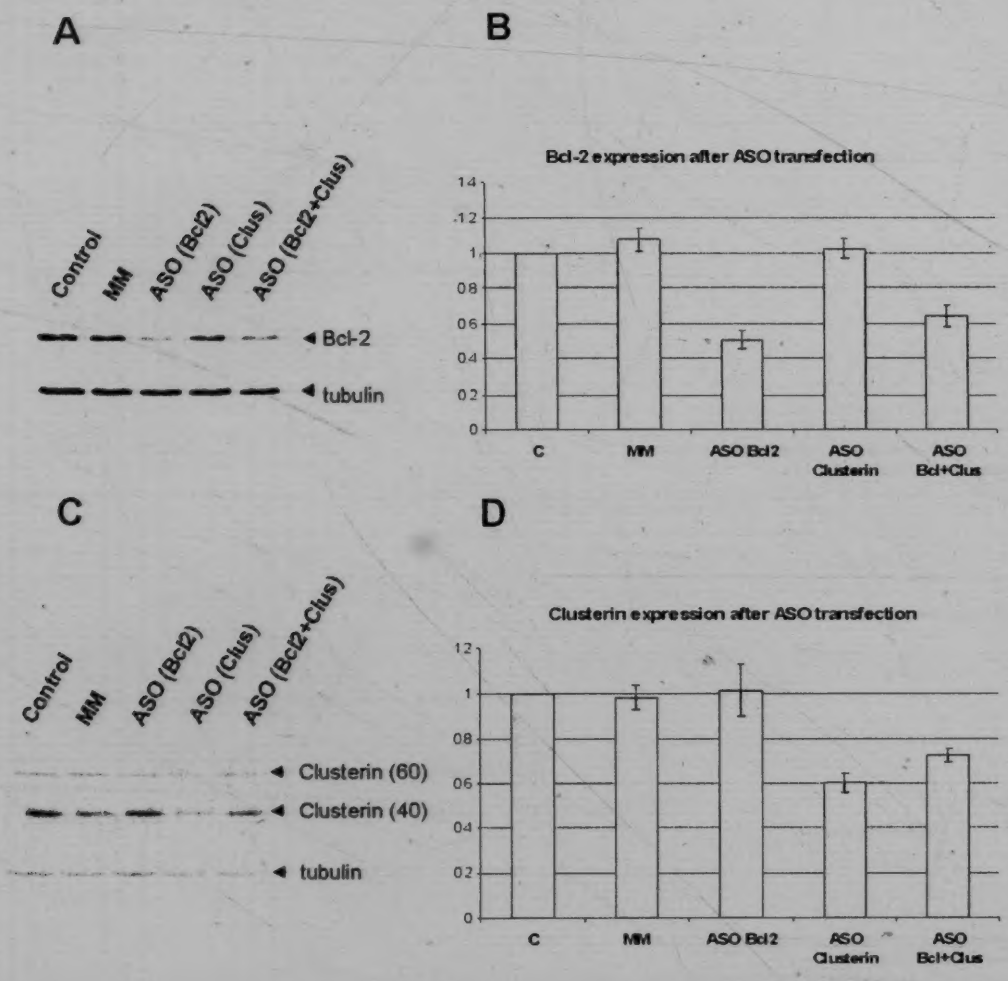


Figure 3.12: Protein expression of Bcl-2 and Clusterin after Bcl-2 ASO combined Clusterin ASO transfection on T24. T24 cells were seeded and cultured overnight. Then the cells were transfected with Bcl-2 ASO, Clusterin ASO and Bcl-2 ASO plus Clusterin ASO at 1000nM for 4 hours. Whole cell proteins were isolated 24 hours after ASO treatment. SDS-PAGE/Western Blots were carried out to determine the protein expression of Bcl-2 and Clusterin (A, C). The quantitative analysis of the protein expression level of Bcl-2 and Clusterin is presented after being normalized to tubulin in B and D.

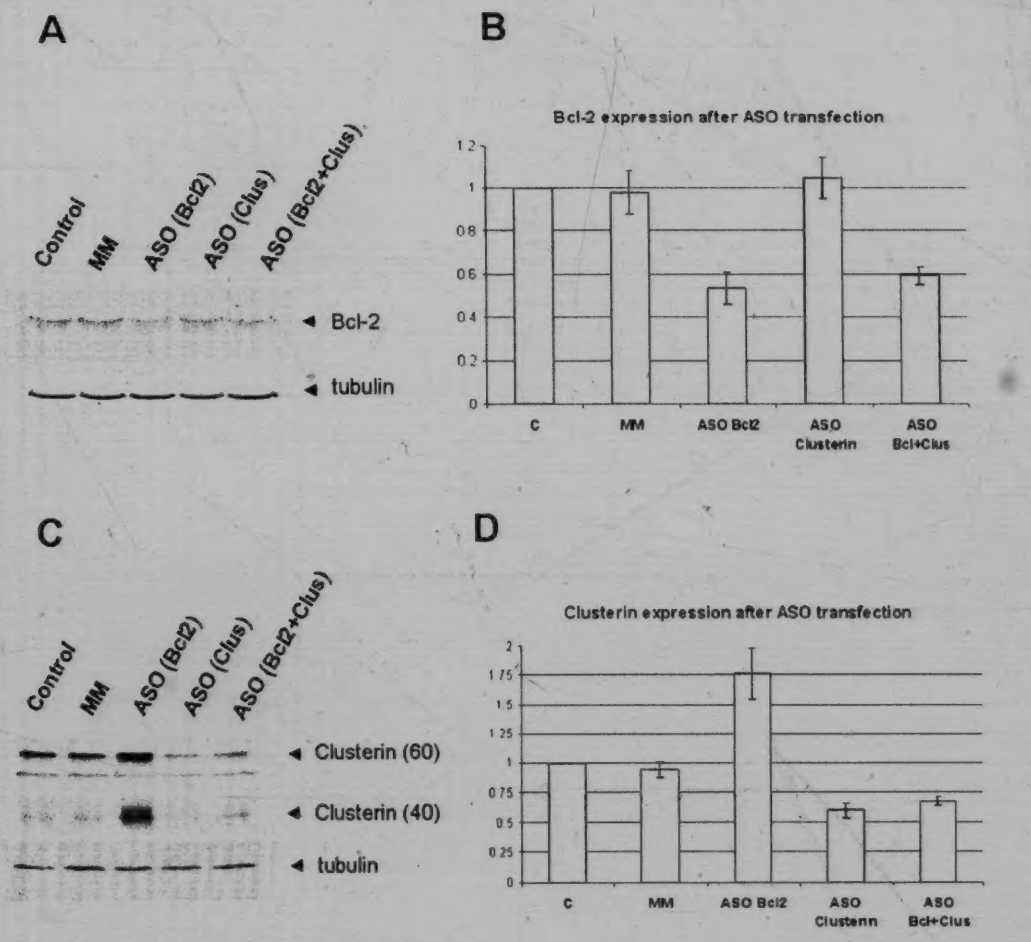


Figure 3.13: Protein expression of Bcl-2 and Clusterin after Bcl-2 ASO combined Clusterin ASO transfection on RT-112. RT-112 cells were seeded and cultured overnight. Cells were then transfected with Bcl-2 ASO, Clusterin ASO and Bcl-2 ASO plus Clusterin ASO at 1000nM for 4 hours. Whole cell proteins were isolated 24 hours after ASO treatment. SDS-PAGE/Western Blots were performed to determine the protein expression of Bcl-2 and Clusterin (A, C). The quantitative analysis of Bcl-2 and Clusterin protein expression level is presented as the average of three times' detections with standard error after normalization to tubulin level (B, D).

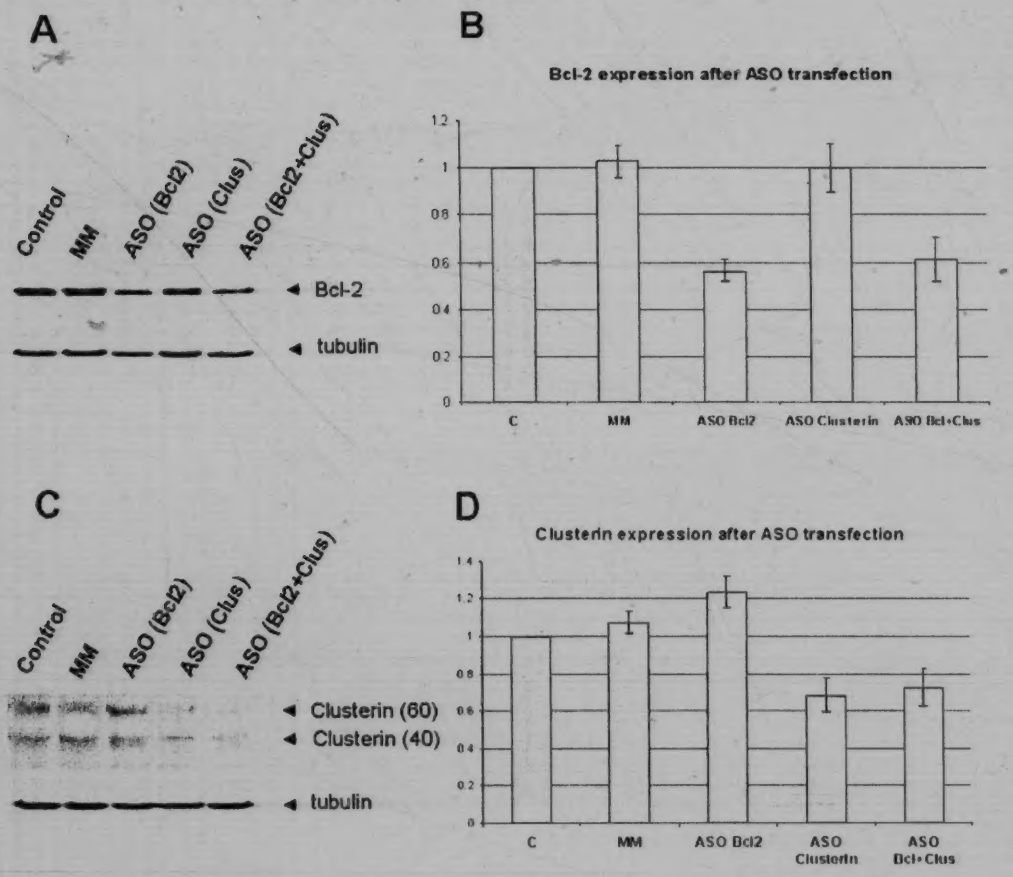


Figure 3.14: Protein expression of Bcl-2 and Clusterin after Bcl-2 ASO combined Clusterin ASO transfection on 253J. 253J cells were seeded and cultured overnight. Twenty-four hours later, the cells were transfected with Bcl-2 ASO, Clusterin ASO and Bcl-2 ASO plus Clusterin ASO at 1000nM for 4 hours, whole cell proteins were isolated. SDS-PAGE/Western Blots were performed to determine the protein expression of Bcl-2 and Clusterin (A, C). The quantitative analysis of the protein expression level of Bcl-2 and Clusterin is shown after normalization to tubulin level (B, D).

shown on the downregulation of Clusterin protein by the transfection of Clusterin ASO alone and combined Bcl-2 ASO and Clusterin ASO transfection. The protein expression level of Clusterin was reduced to 70% compared with untreated cells by the combined transfection of Bcl-2 ASO with Clusterin ASO, which was not as much as with the treatment of Clusterin ASO alone (reduction to 60%) as shown in the Figure 3.12 C and D. The Clusterin protein expression was not affected by the transfection Bcl-2 ASO on T24 cell line. Similar results were also detected on other TRAIL-partially resistant cell lines, RT-112 and 253J (Figure 3.13 and 3.14). Interesting enough, after down-regulating the Bcl-2 protein in another TRAIL-partially resistant cell line RT-112 by Bcl-2 ASO, the protein expression of Clusterin was found to be significantly increased (Figure 3.13 C and D). The upregulation of Clusterin after ASO Bcl-2 treatment was also found on another TRAIL-partially sensitive cell line 253J (Figure 3. 14 C and D), but is not as significant as in RT-112. To examine if the regulation of Bcl-2 and Clusterin through ASO will affect the sensitivity of these TRAIL-partially resistant cells to TRAIL, the ASO combined TRAIL treatments were carried out and are reported in section 3.3

3.3. Effect of ASO Combined TRAIL Treatment on TRAIL-partially Resistant TCCB Cells

3.3.1. Sensitivity of TRAIL-partially Resistant TCCB Cells to TRAIL after ASO Treatment

As indicated in section 3.1.3, the sensitivity of TRAIL-partially resistant TCCB cells to TRAIL seemed to correlate with how much apoptotic signaling transduction has been transferred to the mitochondrial pathway. Thus, the approach to increase the activation of the intrinsic apoptotic signals such as caspase-9 by downregulation of those proteins that prevent mitochondrial release may enhance the TRAIL sensitivity of the TRAIL-partially resistant TCCB cells. To test this hypothesis, ASO combined TRAIL treatments were carried out on those TRAIL-partially resistant TCCB cell lines: T24, RT-112 and 253J. As described in the introduction, the anti-apoptotic function of Bcl-2 and Clusterin is related with their inhibition of mitochondrial release and in turn preventing the activation of caspase-9 and/or caspase-3. In addition, the treatment of 4-hour ASO and 24 hours' culture afterwards can successfully reduce the corresponding protein expression level in TRAIL-partially resistant TCCB cells, T24, RT-112 and 253J. Based on these findings (*vide supra)*, the following ASO combined TRAIL treatments were performed on T24, RT-112, and 253J, TRAIL-partially resistant cell lines. Cells were transfected with Bcl-2 phosphorothioate ASO and/or Clusterin phosphorothioate ASO at 1000nM for 4 hours, with mismatch (MM) oligodeoxynucleotides transfection as a control. After 24 hours, cells were treated with 300ng/ml of recombinant human TRAIL for 4 hours. 20 hours later, MTT assays were carried out to determine the percentage of

cell death. The experiments were repeated at least three times on each cell line. Results are presented as the average values of analyses with standard errors (Figure 3.15-17 A).

As shown in Figure 3.15 A, the Bcl-2 ASO treatment and the Clusterin ASO treatment, as well as the Bcl-2 ASO plus Clusterin ASO treatment, significantly ($p<0.05$) enhanced the sensitivity of T24 cells to TRAIL as compared with the MM (mismatch oligodeoxynucleotides) transfection control. No synergistic effect of Bcl-2 ASO combined with Clusterin ASO treatment was observed on sensitizing T24 cells to TRAIL although the downregulation of protein expression of both Bcl-2 and Clusterin was achieved after cells were transfected with Bcl-2 ASO plus Clusterin ASO. In this study, we also found MM transfection can cause the increase of TRAIL-induced cell death. This phenomenon may be due to the toxicity of phosphorothioate ODN to the cells as observed by other researchers [201]. Furthermore, the Bcl-2 ASO treatment showed the most significant ($p<0.0001$) effect on enhancing TRAIL-induced cell death by 40%, as compared with the treatment of Clusterin ASO alone or Bcl-2 ASO plus Clusterin ASO ($p<0.05$). Similar results were also found in the other TRAIL-partially resistant TCCB cell lines RT-112 and 253J with the exception that Clusterin ASO treatment on 253J cells did not show significantly potentiate TRAIL-induced cell death when compared with MM control (Figure 3.16 A and 3.17 A).

3.3.2. Increase in the Activation of Apoptosis Signaling after ASO Combined TRAIL Treatment on TRAIL-partially Resistant TCCB Cells

As detailed above, the rationale of ASO combined TRAIL strategy is to decrease the protein expression of Bcl-2 and/or Clusterin before TRAIL treatment in order to increase the activation potential of the apoptotic mitochondrial pathway induced by

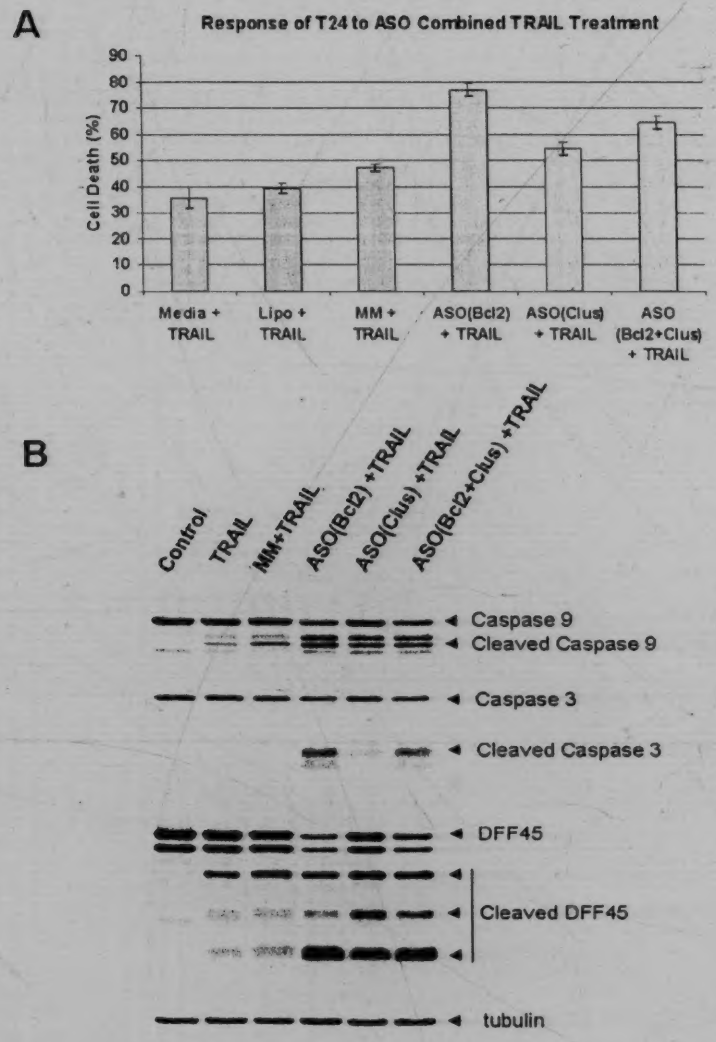


Figure 3.15: Response of T24 to ASO combined TRAIL treatments. Cells were seeded and transfected with 1000nM of Bcl-2 ASO and/or Clusterin ASO and MM for 4 hours. After 24 hours, cells were treated with 300ng/ml of recombinant human TRAIL for 4 hours. 20 hours later, MTT assays were performed to determine the percentage of cell death. Results are presented as the average value with standard error (A). Proteins were isolated immediately after 4 hours of TRAIL exposure. The apoptotic signals were detected through Western Blotting with corresponding antibodies (B).

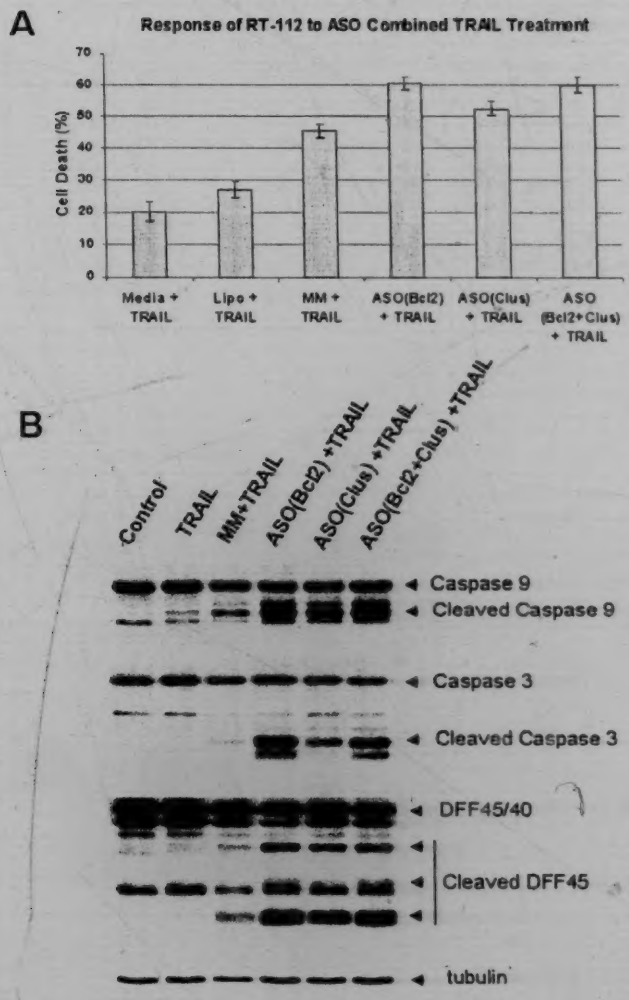


Figure 3.16: Response of RT-112 to ASO combined TRAIL treatments. Cells were seeded and transfected with Bcl-2 ASO and/or Clusterin ASO and MM at 1000nM for 4 hours. 24 hours later, cells were treated with 300ng/ml of recombinant human TRAIL for 4 hours. After 20 hours, MTT assays were performed to determine the percentage of cell death (A). Proteins were isolated immediately after TRAIL treatments. The apoptotic signals were detected through SDS-PAGE/Western Blots (B).

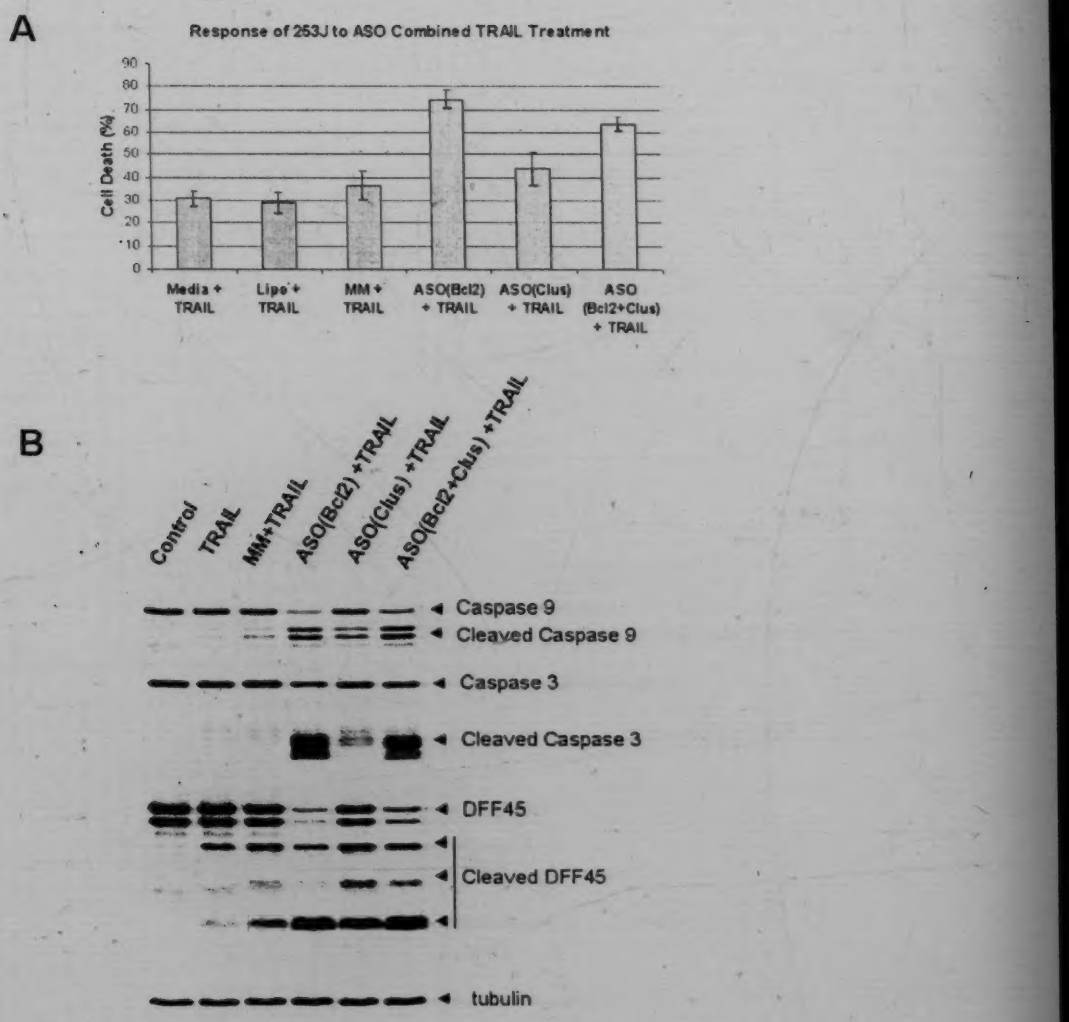


Figure 3.17: Response of 253J to ASO combined TRAIL treatments. Cells were seeded and transfected with Bcl-2 ASO and/or Clusterin ASO and MM at 1000nM for 4 hours. After 24 hours, cells were treated with 300ng/ml of recombinant human TRAIL for 4 hours. 20 hours later, MTT assays were then carried out to determine the percentage of cell death (A). Proteins were isolated right immediately following TRAIL treatments. The apoptotic signals were detected through SDS-PAGE/Western Blots (B).

TRAIL. To further determine the apoptotic signal activation after ASO combined TRAIL treatment, TRAIL-partially resistant TCCB cells (T24, RT-112 and 253J) were transfected with 1000nM of Bcl-2 ASO and/or Clusterin ASO for 4 hours. After 24 hours, cells were treated with recombinant human TRAIL at 300ng/ml for 4 hours with untreated cells as a negative control. In order to catch the apoptotic signal transduction, proteins were isolated immediately following TRAIL treatment. The apoptotic signals were then detected through Western blotting as shown in Figure 3.15B-3.17 B.

As presented in Figure 3.15 B, the cleaved caspase-9, caspase-3, and DFF45 were successfully detected after TRAIL treatment as well as ASO combined TRAIL treatment on T24 cells. The significant increase in the cleaved active caspase-9 and decrease in the full length caspase-9 (proenzyme) delineate the role of downregulation of Bcl-2 and/or Clusterin on this TRAIL-induced mitochondrial pathway. The increased cleavage of downstream apoptotic signals, caspase-3 and DFF45 were consistent with the TRAIL sensitivity detection through MTT assay. The increased apoptotic signaling on the mitochondrial pathway were also detected on the other TRAIL-partially resistant TCCB cell lines RT-112 and 253J after ASO combined TRAIL treatments (Figure 3.16 B and 3.17 B).

3.3.3. Live/Dead Assay

The effects of ASO combined TRAIL treatment on TRAIL-partially resistant cells were also observed through the Live/Dead assay. After the same treatment schedule described in section 3.3.1 (4-hour ASO transfection—24-hour incubation—4-hour TRAIL treatment—24-hour incubation), cells were treated with 7.5μM of live cell nucleic stain Syto Green 16 and 2.5μM of dead cell stain Propidium Iodide (PI) for 1

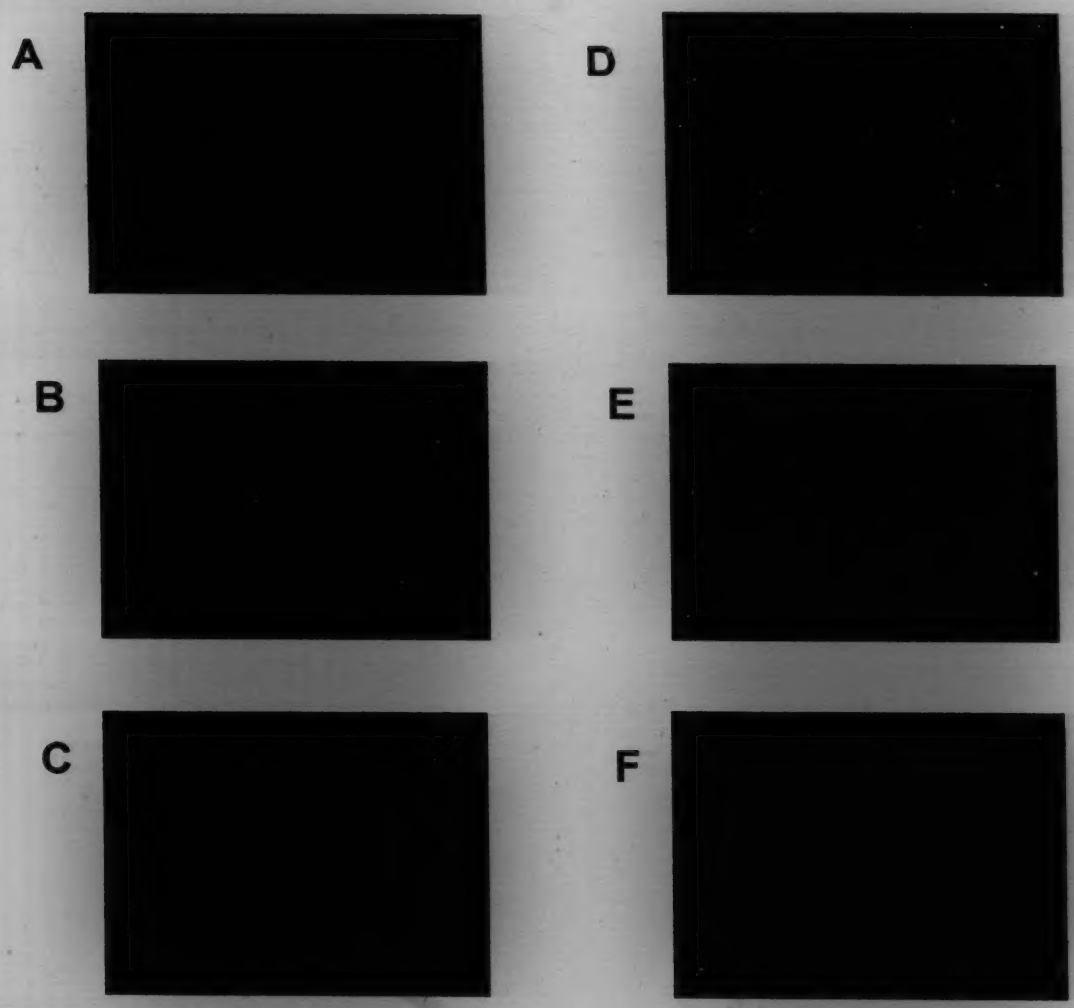


Figure 3.18: Live/Dead assay to detect the response of RT-112 to ASO combined TRAIL treatments. Cells were seeded and transfected with Bcl-2 ASO (D), Clusterin ASO (E), Bcl-2 ASO plus Clusterin ASO (F) and MM oligodeoxynucleotides (C) at 1000nM for 4 hours with untreated (A) and untransfected cells (B) as controls. 24 hours later, cells were treated with 300ng/ml of recombinant human TRAIL for 4 hours except for the untreated cells as the control (A). After 24 hours, cells were stained with 7.5μM of live cell nucleic stain Syto16 and 2.5μM of dead cell stain Propidium Iodide (PI) for 1 hour at 37°C. Dual fluorescence images were analyzed by fluorescence microscopy.

hour at 37°C. Dual fluorescence images were analyzed by fluorescence microscopy.

As shown in Figure 3.18, the observed results were similar to the ones detected by MTT assay. The Bcl-2 ASO treatment alone or when combined with Clusterin ASO, was found to increase TRAIL-induced cell death the most, as compared with the other treatments on TRAIL-partially resistant TCCB cells RT-112. Although it is hard to identify from the images which treatment, between the Bcl-2 ASO transfection alone and the transfection of Bcl-2 ASO plus Clusterin ASO, is more significant in enhancing TRAIL-induced cell death, it is further evidence to support the promising potential of TRAIL-ASO combination therapy in future TCCB treatment.

Chapter 4: Conclusion and Discussion

Transitional Cell Carcinoma of the Bladder (TCCB) is the 2nd most common urologic neoplasm and the 12th leading cause of cancer deaths. The majority of TCCB are superficial with a significant risk of recurrence. Intravesical therapies are usually required after initial endoscopic surgical removal, due to the high probability of recurrence and progression of TCCB. Although conventional intravesical BCG therapy appears to have the best treatment response to date, the serious side effects due to its potential infectivity and non-specific cytokine response necessitate a better intravesical strategy. Compelling evidence suggest that TRAIL may have the potential for clinical application as a novel anti-TCCB agent, since it can selectively induce apoptosis in most tumor and transformed cells, but not in normal cells [89-91]. Moreover, TRAIL is believed to be one of the mechanisms for BCG induced anti-tumor activity in the treatment of bladder cancer [8]. Thus, the aim of this study was to explore the application of TRAIL and develop in a rational fashion, a novel intravesical molecular strategy for the treatment of superficial TCCB that would optimally induce apoptosis. As the anti-apoptotic functions of Bcl-2 and Clusterin have been demonstrated in many human malignancies [148,156,157,164], in addition to exploring the response of TCCB cells to TRAIL, the translational research presented in this thesis has further examined the effect of TRAIL in combination with Bcl-2 ASO and/or Clusterin ASO treatments on TRAIL-partially resistant TCCB cells.

4.1. TCCB Cells Exhibit Differential Response to Recombinant Human TRAIL

Although TRAIL-induced apoptosis has been well studied in several malignant cells, the response of TCCB cells to TRAIL has not been systematically investigated. In this study, we found that the recombinant human TRAIL triggered apoptosis of TCCB cells in a dose and time dependent manner. The efficiency of TRAIL-induced apoptosis in different TCCB cell lines varied significantly, leading us to characterize those cell lines as sensitive [RT-4, UMUC14, HTB-9 (5637), MGH-U3 and UMUC3], partially resistant [RT-112, 253J, UMUC9, T24 and UMUC6] and resistant [CRL-1472 (HT-1376)] to TRAIL-induced cytotoxicity (apoptosis) based on the percentage of cell death. This differential response to TRAIL may be due in part to the expression of death receptors and decoy receptors on the cell surface and/or the ratio of pro- and anti-apoptotic proteins in the cells. The DcR1 and DcR2 has been proposed to inhibit TRAIL induced apoptosis either by competitive binding of TRAIL or by providing inhibitory signals such as activation of the transcription factor NF-κB that is known to regulate the anti-apoptotic proteins expression, for example, IAPs [107,216,217].

In addition, we found that both caspase-8 and caspse-9 were activated in response to TRAIL treatment, indicating that TRAIL triggers not only death receptor but also mitochondrial apoptotic pathways in these TCCB cells. Supportive of this, we showed that TRAIL triggered Bid activation by the way of activated caspase-8 in these cells (see details in section 3.1.2.), which mediates the crosstalk between death-receptor pathway and mitochondrial pathway. The detection of active caspase-3 and cleaved DFF45 (ICAD) further demonstrated the characteristic of this TRAIL-induced cell killing in TCCB cells. As demonstrated in Figure 3.2, our data show that in most of TRAIL responsive TCCB cells, the apoptotic signaling did not start until the TRAIL exposure

was longer than 2 hours. However, in the most TRAIL-sensitive TCCB cell line UMUC14, the initiation of the apoptotic signals was started earlier than the other cell lines, i.e. after 1 hour TRAIL treatment. On the other hand, in TRAIL-resistant TCCB cell line CRL-1472 (HT-1376), no activated signal was detected even after treatment with 1000ng/ml of TRAIL for 24 hours. The differential response of TCCB cells suggests the presence of a complicated regulatory mechanism in TRAIL-induced apoptosis. Considering the absence of the activation of caspase-8 (the initiator caspase on the death receptor pathway) in response to TRAIL, the resistance of CRL-1472 to TRAIL-induced apoptosis might be associated to the relative expression level of TRAIL death receptors and TRAIL decoy receptors on the surface or their trimerization after the TRAIL binding [100], the absent or abnormal adaptor FADD [125], or the over-expression of c-FLIP [218]. Most recently, it has been reported that the induction of DISC assembly in lipid rafts of plasma membrane is critical for the activation of caspase-8 in TRAIL-induced apoptosis. Moreover, the cFLIP was found to mediate the DISC assembly in nonrafts, and the knockdown of cFLIP has been shown to redistribute the DISC from nonrafts to lipid rafts, thereby activating the caspase-8 initiated apoptosis [219]. Thus regulation of cFLIP might be one possible approach to sensitize the TRAIL-resistant TCCB cells CRL-1472 to TRAIL.

In TRAIL-partially resistant cells, the activation of Bid was demonstrated and further shown to be correlated with their TRAIL sensitivity. This is significant as this result strongly suggests the dependency of TRAIL-induced apoptosis of TCCB cells on the mitochondrial pathway, which is also consistent with the previous findings in other malignancies (e.g. lung cancer and melanoma) [156,157]. Therefore, their partial

resistance to TRAIL might be related to the regulatory factors on the intrinsic apoptotic pathway, such as overexpression of anti-apoptotic proteins [93,100], and hence TRAIL alone may have its limitation in tumor clearance as an anti-TCCB reagent. Recently, several studies have shown that combining chemotherapy with the aim to downregulate the expression of such anti-apoptotic genes appears to be a more rational strategy to enhance the chemosensitivity than the conventional strategy of increasing the dose and combining several kinds of drugs for the treatment of cancer [148,188]. To overcome the resistance to TRAIL in some TCCB cells, a translational study of combination therapies is being conducted for clinical application in the Moore lab. This work represents a natural extension to previous novel therapies to induce selective cell death in TCCB.

4.2. Recombinant Human TRAIL is not Toxic to Normal Cells

The safety of application and delivery of anti-cancer reagents is another concern of novel therapies. Although TNF and Fas ligand also can induce apoptosis in tumor cells, their potential clinical usage was rapidly abandoned due to their significant toxicities to normal tissues [87,88]. The selected killing of TRAIL makes it the best candidate as the potential anti-TCCB agent. Various versions of recombinant human TRAIL have been generated to date and shown differential toxicity on the normal cells. Currently, the latest version of the non-tagged ligands that contains amino acids 114-281 of human Apo2L/TRAIL has been optimised and tested on isolated human hepatocytes and animal models by several investigators. It has been found that the non-tagged optimised version of TRAIL has a profound apoptotic effect on tumor cells but is nontoxic to human liver cells in vitro and in vivo [220-222].

In this study, the safety of using recombinant human TRAIL as a potential intravesical agent was examined through MTT assays and Western blots on the normal fibroblast cells F2P6, as well as the Live/Dead assays on the co-cultured spheroids of human TCCB cells MGH-U3 and normal fibroblast cells F2P6. The normal fibroblast cells F2P6 did not show any response to recombinant human TRAIL even when the applied TRAIL concentration was escalated to as high as 1000ng/ml with exposure time as long as 24 hours. The absence of active apoptotic signals after TRAIL treatment was further confirmed through Western blots showing that human recombinant TRAIL does not trigger apoptosis in normal F2P6 cells. Moreover, the selective killing of TCCB cells MGH-U3 with sparing of normal cells F2P6 was found on the topical application of recombinant human TRAIL in our *in vitro* TCCB model (co-cultured MGH-U3/F2P6 spheroids). The lack of response of normal cells to TRAIL is consistent with Ashkenazi's previous study on the safety of recombinant human TRAIL as determined on various primary normal human cell types [7]. Neither morphological evidence of apoptosis, nor change in staining of the cells by propidium iodide has been found after early-passage primary human umbilical vein endothelial cells; lung fibroblasts; mammary, renal, or prostate epithelial cells; colon smooth muscle cells; or astrocytes were exposed to Apo2L/TRAIL (1μg/ml) for 24 hours. The research described above supports that human recombinant TRAIL has potential as an intravesical reagent to promote apoptosis and clearance of TCCB with minimal toxicity.

4.3. ASO Treatment Sensitizes TRAIL-partially Resistant Cells to TRAIL

As indicated in section 4.1, TCCB cell lines showed differential sensitivity to TRAIL, i.e. sensitive, partially resistant and total resistant. The underlying molecular mechanism of the resistance to apoptosis in the TRAIL-partially resistant cell lines could be any of the molecules involved in the death receptor pathway and/or mitochondrial pathway induced by TRAIL. As presented in section 3.1.3, the cleaved Bid appeared to correlate with the TRAIL sensitivity in the TRAIL-partially resistant cell lines. This relationship might be due to the difference in the amounts of activated caspase-8 induced by TRAIL and/or the variant amounts of tBid cleaved by active caspase-8 in the cells. Nevertheless, the low level of apoptotic signaling on mitochondrial pathway could be one of the crucial mechanisms for the TRAIL resistance of these TRAIL-partially resistant TCCB cells. So increasing the activation of Bid or decreasing the inhibition of mitochondrial apoptotic signaling transduction might sensitize the TRAIL-partially resistant cells to TRAIL. It was reported that the selective downregulation of cFLIP or receptor-interacting protein (RIP) could activate caspase-8 to sensitize resistant cells to TRAIL, thus it might be an approach to increase the Bid activation and tBid-mediated mitochondrial apoptotic pathway [219]. In our study, based on previous studies about the anti-apoptotic functions of Bcl-2 and Clusterin on the mitochondria, downregulation of these proteins could be one of the approaches to sensitize the TRAIL-partially resistant cells to TRAIL by increasing the activation of TRAIL-induced apoptotic signals on mitochondrial pathway. Therefore, the treatments of TRAIL combined with ASO were carried out on three TRAIL-partially resistant cell lines T24, RT-112 and 253J as presented in section 3.3.

After downregulation of Bcl-2 was achieved following 4-hour transfection of Bcl-2 ASO on T24, RT-112 and 253J cell lines, the percentage of cell death induced by TRAIL was significantly increased by 40% as compared with the untransfected TRAIL-treated cells. This was associated with the significant increase in the activation of caspase-9, caspase-3 and cleaved DFF45, and the decrease in full length of these molecules detected through Western blots, suggesting the essential role of the mitochondrial pathway in the TRAIL-mediated apoptosis in TCCB.

The transfection of TCCB cells with Clusterin ASO enhanced the TRAIL-induced cell death in T24 and RT-112 cells, but not 253J cells. This might be due to the fact that 253J cells have higher basal Clusterin protein expression compared with T24 and RT-112 cells, as shown in Figure 3.7. Since the transfection of phosphorothioate antisense oligodeoxynucleotides can just reduce protein expression by ~55% (as presented in section 3.2), the protein expression of Clusterin after the transfection with Clusterin ASO in 253J cells may not be low enough to abrogate its anti-apoptotic function, suggesting that the downregulation of Clusterin may need to reach a certain or critical level in order to decrease its anti-apoptotic function. Thus, more efficient ways to decrease or knockdown Clusterin protein on those high Clusterin expressing cancer cells may be needed to reduce its anti-apoptotic function. The Clusterin-specific small interfering RNA (siRNA) oligonucleotides have been demonstrated to potentially silence *Clusterin* gene expression, which might prove to be a valuable agent to enhance the TRAIL sensitivity of those TRAIL-partially resistant TCCB cells [223].

As discussed above, antisense ODN to either Bcl-2 or Clusterin decrease their respective protein expression and consequently increase TRAIL sensitivity in TCCB cells.

When both Bcl-2 ASO and Clusterin ASO were combined together, although there was reduction in both protein levels, the expected synergy in enhancing sensitivity to TRAIL was not found in TCCB cells. On the other hand, the effects were found to be even lower than Bcl-2 ASO transfection alone. This might be because the reduction of Bcl-2 protein by Bcl-2 ASO and Clusterin ASO combined transfection was not as much as Bcl-2 ASO alone (described in the section 3.2), suggesting that downregulation of Bcl-2 by Bcl-2 ASO has the most potent function on increasing the TRAIL sensitivity of TRAIL-partially resistant TCCB cell lines.

4.4 Concluding Remarks

The work of this thesis has demonstrated that TRAIL can selectively trigger apoptosis in TCCB cells through both death receptor pathway and tBid mediated mitochondrial pathway, and leave the normal cells intact. Our study also demonstrated the efficiency of TRAIL-induced apoptosis in TCCB cell lines is variable and five out of eleven show partial resistances to TRAIL. Additionally, we found that the amount of Bid cleavage appeared to be correlated with the sensitivity of TCCB cells to TRAIL in the TRAIL partially-resistant cells, strongly suggesting a crucial role of tBid-mediated mitochondrial pathway in the TRAIL-induced apoptosis of TCCB cells. Consistent with this, we further demonstrated that downregulation of Bcl-2 by antisense oligodeoxynucleotides can significantly sensitize these TCCB cells to TRAIL. The work presented in this study is the first step of the on-going translational research in our laboratory, providing evidence that the TRAIL-ASO combination strategy has significant promise as a novel intravesical therapy for TCCB treatment. Another study exploring the

effect of Gemzar on TRAIL-induced apoptosis in TCCB cells is currently underway (work of Jacob Moibi), and has led to some very significant findings that support these observations and those of others. This study has shown that Gemzar chemotherapy reduces Bcl-2 expression and thereby potentiates TRAIL induced apoptosis. With this proof of principle we have initiated further studies in our TCCB animal models to determine if TRAIL in combinations with ASO and/or Gemzar will eliminate TCCB *in vivo* with few side effects. Hopefully, these translational studies taken together would become a novel therapeutic strategy for TCCB treatment in the future.

Chapter 5: Bibliography

1. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward A, Ward E, Feuer EJ, Thun MJ: Cancer Statistics. *CA Cancer J Clin* 2004, 54:8-29.
2. Grossman HB: Superficial bladder cancer: decreasing the risk of recurrence. *Oncology (Williston Park)* 1996, 10:1617-1624; discussion 1624, 1627-1618.
3. Hurle R, Losa A, Manzetti A, Lembo A: Intravesical bacille Calmette-Guerin in Stage T1 grade 3 bladder cancer therapy: a 7-year follow-up. *Urology* 1999, 54:258-263.
4. Dalbagni G, Herr HW: Current use and questions concerning intravesical bladder cancer group for superficial bladder cancer. *Urol Clin North Am* 2000, 27:137-146.
5. Lamm DL, van der Meijden AP, Akaza H, Brendler C, Hedlund PO, Mizutani Y, Ratliff TL, Robinson MR, Shinka T: Intravesical chemotherapy and immunotherapy: how do we assess their effectiveness and what are their limitations and uses? *Int J Urol* 1995, 2 Suppl 2:23-35.
6. Luo Y, Chen X, Han R, O'Donnell MA: Recombinant bacille Calmette-Guerin (BCG) expressing human interferon-alpha 2B demonstrates enhanced immunogenicity. *Clin Exp Immunol* 2001, 123:264-270.
7. Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, et al.: Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999, 104:155-162.
8. Ludwig AT, Moore JM, Luo Y, Chen X, Saltsgaver NA, O'Donnell MA, Griffith TS: Tumor necrosis factor-related apoptosis-inducing ligand: a novel mechanism for Bacillus Calmette-Guerin-induced antitumor activity. *Cancer Res* 2004, 64:3386-3390.
9. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A: Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996, 271:12687-12690.
10. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA, et al.: Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995, 3:673-682.
11. Cha SS, Kim MS, Choi YH, Sung BJ, Shin NK, Shin HC, Sung YC, Oh BH: 2.8 A resolution crystal structure of human TRAIL, a cytokine with selective antitumor activity. *Immunity* 1999, 11:253-261.
12. Mariani SM, Krammer PH: Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. *Eur J Immunol* 1998, 28:1492-1498.
13. Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A: Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* 2000, 12:611-620.
14. Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME: FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *Embo J* 1997, 16:2794-2804.
15. Hersey P, Zhang XD: How melanoma cells evade trail-induced apoptosis. *Nat Rev Cancer* 2001, 1:142-150.

16. Liu X, Zou H, Slaughter C, Wang X: DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 1997, 89:175-184.
17. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X: Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998, 94:481-490.
18. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997, 91:479-489.
19. Mostofi FK, Davis CJ, Sesterhenn IA: Pathology of tumors of the urinary tract. In: Skinner DG, Lieskovsky G, eds.: Diagnosis and Management of Genitourinary Cancer. Philadelphia, Pa: WB Saunders. 1988:83-117.
20. Oxford G, Theodorescu D: The role of Ras superfamily proteins in bladder cancer progression. *J Urol* 2003, 170:1987-1993.
21. Kimura T, Suzuki H, Ohashi T, Asano K, Kiyota H, Eto Y: The incidence of thanatophoric dysplasia mutations in FGFR3 gene is higher in low-grade or superficial bladder carcinomas. *Cancer* 2001, 92:2555-2561.
22. Jimenez RE, Hussain M, Bianco FJ, Jr., Vaishampayan U, Tabazcka P, Sakr WA, Pontes JE, Wood DP, Jr., Grignon DJ: Her-2/neu overexpression in muscle-invasive urothelial carcinoma of the bladder: prognostic significance and comparative analysis in primary and metastatic tumors. *Clin Cancer Res* 2001, 7:2440-2447.
23. van Rhijn BW, Lurkin I, Radvanyi F, Kirkels WJ, van der Kwast TH, Zwarthoff EC: The fibroblast growth factor receptor 3 (FGFR3) mutation is a strong indicator of superficial bladder cancer with low recurrence rate. *Cancer Res* 2001, 61:1265-1268.
24. van Rhijn BW, van Tilborg AA, Lurkin I, Bonaventure J, de Vries A, Thiery JP, van der Kwast TH, Zwarthoff EC, Radvanyi F: Novel fibroblast growth factor receptor 3 (FGFR3) mutations in bladder cancer previously identified in non-lethal skeletal disorders. *Eur J Hum Genet* 2002, 10:819-824.
25. Smith ND, Rubenstein JN, Eggener SE, Kozlowski JM: The p53 tumor suppressor gene and nuclear protein: basic science review and relevance in the management of bladder cancer. *J Urol* 2003, 169:1219-1228.
26. Boice JD, Jr., Engholm G, Kleinerman RA, Blettner M, Stovall M, Lisco H, Moloney WC, Austin DF, Bosch A, Cookfair DL, et al.: Radiation dose and second cancer risk in patients treated for cancer of the cervix. *Radiat Res* 1988, 116:3-55.
27. Johnstone PA, Powell CR, Riffenburgh R, Rohde DC, Kane CJ: Second primary malignancies in T1-3N0 prostate cancer patients treated with radiation therapy with 10-year followup. *J Urol* 1998, 159:946-949.
28. Heney NM, Ahmed S, Flanagan MJ, Frable W, Corder MP, Hafermann MD, Hawkins IR: Superficial bladder cancer: progression and recurrence. *J Urol* 1983, 130:1083-1086.
29. Heney NM, Proppe K, Prout GR, Jr., Griffin PP, Shipley WU: Invasive bladder cancer: tumor configuration, lymphatic invasion and survival. *J Urol* 1983, 130:895-897.

30. Knowles MA: Molecular subtypes of bladder cancer: Jekyll and Hyde or chalk and cheese? *Carcinogenesis* 2006, 27:361-373.
31. Kiemeney LA, Witjes JA, Verbeek AL, Heijbroek RP, Debruyne FM: The clinical epidemiology of superficial bladder cancer. Dutch South-East Cooperative Urological Group. *Br J Cancer* 1993, 67:806-812.
32. Fair WR, Fuks ZY, Scher HI: Cancer of the bladder, in Devita VT, Hellman S, Rosenberg SA (eds): Cancer: Principles and Practices of Oncology, Philadelpha, JB LIppincott. 1993:1052-1072.
33. Bostwick DG: Natural history of early bladder cancer. *J Cell Biochem Suppl* 1992, 16I:31-38.
34. Lamm DL: Long-term results of intravesical therapy for superficial bladder cancer. *Urol Clin North Am* 1992, 19:573-580.
35. Pawinski A, Sylvester R, Kurth KH, Bouffioux C, van der Meijden A, Parmar MK, Bijnens L: A combined analysis of European Organization for Research and Treatment of Cancer, and Medical Research Council randomized clinical trials for the prophylactic treatment of stage TaT1 bladder cancer. European Organization for Research and Treatment of Cancer Genitourinary Tract Cancer Cooperative Group and the Medical Research Council Working Party on Superficial Bladder Cancer. *J Urol* 1996, 156:1934-1940, discussion 1940-1931.
36. Lamm DL, Blumenstein BA, Crawford ED, Montie JE, Scardino P, Grossman HB, Stanisic TH, Smith JA, Jr., Sullivan J, Sarosdy MF, et al.: A randomized trial of intravesical doxorubicin and immunotherapy with bacille Calmette-Guerin for transitional-cell carcinoma of the bladder. *N Engl J Med* 1991, 325:1205-1209.
37. Krege S, Giani G, Meyer R, Otto T, Rubben H: A randomized multicenter trial of adjuvant therapy in superficial bladder cancer: transurethral resection only versus transurethral resection plus mitomycin C versus transurethral resection plus bacillus Calmette-Guerin. Participating Clinics. *J Urol* 1996, 156:962-966.
38. Herr HW, Laudone VP, Badalament RA, Oettgen HF, Sogani PC, Freedman BD, Melamed MR, Whitmore WF, Jr.: Bacillus Calmette-Guerin therapy alters the progression of superficial bladder cancer. *J Clin Oncol* 1988, 6:1450-1455.
39. Morales A, Eidinger D, Bruce AW: Intracavitary bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *J Urol* 1976, 116:180-183.
40. Ratliff TL, Palmer JO, McGarr JA, Brown EJ: Intravesical Bacillus Calmette-Guerin therapy for murine bladder tumors: initiation of the response by fibronectin-mediated attachment of Bacillus Calmette-Guerin. *Cancer Res* 1987, 47:1762-1766.
41. Bevers RFM, Boer ECd, Kurth K-H, Schamhart DHJ: BCG internalization in human bladder cancer cell lines, especially with regard to cell surface-expressed fibronection. *Aktuelle Urol* 2000, 31 (suppl 1):31-34.
42. Durek C, Brandau S, Ulmer AJ, Flad HD, Jocham D, Bohle A: Bacillus-Calmette-Guerin (BCG) and 3D tumors: an in vitro model for the study of adhesion and invasion. *J Urol* 1999, 162:600-605.

43. Lattime EC, Gomella LG, McCue PA: Murine bladder carcinoma cells present antigen to BCG-specific CD4+ T-cells. *Cancer Res* 1992, 52:4286-4290.
44. Neyrolles O, Gould K, Gares MP, Brett S, Janssen R, O'Gaora P, Herrmann JL, Prevost MC, Perret E, Thole JE, et al.: Lipoprotein access to MHC class I presentation during infection of murine macrophages with live mycobacteria. *J Immunol* 2001, 166:447-457.
45. Alexandroff AB, Jackson AM, O'Donnell MA, James K: BCG immunotherapy of bladder cancer: 20 years on. *Lancet* 1999, 353:1689-1694.
46. Kawashima T, Norose Y, Watanabe Y, Enomoto Y, Narazaki H, Watari E, Tanaka S, Takahashi H, Yano I, Brenner MB, et al.: Cutting edge: major CD8 T cell response to live bacillus Calmette-Guerin is mediated by CD1 molecules. *J Immunol* 2003, 170:5345-5348.
47. Bevers RF, de Boer EC, Kurth KH, Schamhart DH: BCG-induced interleukin-6 upregulation and BCG internalization in well and poorly differentiated human bladder cancer cell lines. *Eur Cytokine Netw* 1998, 9:181-186.
48. Lauta VM: A review of the cytokine network in multiple myeloma: diagnostic, prognostic, and therapeutic implications. *Cancer* 2003, 97:2440-2452.
49. Lamm DL: Complications of bacillus Calmette-Guerin immunotherapy. *Urol Clin North Am* 1992, 19:565-572.
50. Lamm DL, van der Meijden PM, Morales A, Brosman SA, Catalona WJ, Herr HW, Soloway MS, Steg A, Debruyne FM: Incidence and treatment of complications of bacillus Calmette-Guerin intravesical therapy in superficial bladder cancer. *J Urol* 1992, 147:596-600.
51. Herr HW: Tumor progression and survival of patients with high grade, noninvasive papillary (TaG3) bladder tumors: 15-year outcome. *J Urol* 2000, 163:60-61; discussion 61-62.
52. Kerr JF, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972, 26:239-257.
53. Okada H, Mak TW: Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* 2004, 4:592-603.
54. Nicholson DW, Thornberry NA: Caspases: killer proteases. *Trends Biochem Sci* 1997, 22:299-306.
55. Salvesen GS, Dixit VM: Caspases: intracellular signaling by proteolysis. *Cell* 1997, 91:443-446.
56. Thornberry NA, Lazebnik Y: Caspases: enemies within. *Science* 1998, 281:1312-1316.
57. Howard AD, Chartrain N, Ding GF, Kostura MJ, Limjuco G, Schmidt JA, Tocci MJ: Probing the role of interleukin-1 beta convertase in interleukin-1 beta secretion. *Agents Actions Suppl* 1991, 35:77-83.
58. Earnshaw WC, Martins LM, Kaufmann SH: Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 1999, 68:383-424.
59. Cohen GM: Caspases: the executioners of apoptosis. *Biochem J* 1997, 326 (Pt 1):1-16.
60. Stennicke HR, Salvesen GS: Properties of the caspases. *Biochim Biophys Acta* 1998, 1387:17-31.

61. Fuentes-Prior P, Salvesen GS: The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 2004, 384:201-232.
62. Degterev A, Boyce M, Yuan J: A decade of caspases. *Oncogene* 2003, 22:8543-8567.
63. Hengartner MO: The biochemistry of apoptosis. *Nature* 2000, 407:770-776.
64. Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM: An induced proximity model for caspase-8 activation. *J Biol Chem* 1998, 273:2926-2930.
65. Porter AG, Janicke RU: Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999, 6:99-104.
66. Sakahira H, Enari M, Nagata S: Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 1998, 391:96-99.
67. Buendia B, Santa-Maria A, Courvalin JC: Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. *J Cell Sci* 1999, 112 (Pt 11):1743-1753.
68. Bratton SB, Cohen GM: Apoptotic death sensor: an organelle's alter ego? *Trends Pharmacol Sci* 2001, 22:306-315.
69. Schulze-Osthoff K, Krammer PH, Droge W: Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. *Embo J* 1994, 13:4587-4596.
70. Kaptein A, Jansen M, Dilaver G, Kitson J, Dash L, Wang E, Owen MJ, Bodmer JL, Tschopp J, Farrow SN: Studies on the interaction between TWEAK and the death receptor WSL-1/TRAMP (DR3). *FEBS Lett* 2000, 485:135-141.
71. Bratton SB, MacFarlane M, Cain K, Cohen GM: Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. *Exp Cell Res* 2000, 256:27-33.
72. Chinnaiyan AM, O'Rourke K, Yu GL, Lyons RH, Garg M, Duan DR, Xing L, Gentz R, Ni J, Dixit VM: Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. *Science* 1996, 274:990-992.
73. Boldin MP, Goncharov TM, Goltsev YV, Wallach D: Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 1996, 85:803-815.
74. Kimberley FC, Screaton GR: Following a TRAIL: update on a ligand and its five receptors. *Cell Res* 2004, 14:359-372.
75. Green DR, Reed JC: Mitochondria and apoptosis. *Science* 1998, 281:1309-1312.
76. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X: Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997, 90:405-413.
77. Du C, Fang M, Li Y, Li L, Wang X: Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 2000, 102:33-42.
78. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL: Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000, 102:43-53.
79. Darmon AJ, Nicholson DW, Bleackley RC: Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* 1995, 377:446-448.

80. Atkinson EA, Barry M, Darmon AJ, Shostak I, Turner PC, Moyer RW, Bleackley RC: Cytotoxic T lymphocyte-assisted suicide. Caspase 3 activation is primarily the result of the direct action of granzyme B. *J Biol Chem* 1998, 273:21261-21266.
81. Ashkenazi A, Dixit VM: Death receptors: signaling and modulation. *Science* 1998, 281:1305-1308.
82. Ashkenazi A: Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002, 2:420-430.
83. Caldwell SA, Ryan MH, McDuffie E, Abrams SI: The Fas/Fas ligand pathway is important for optimal tumor regression in a mouse model of CTL adoptive immunotherapy of experimental CMS4 lung metastases. *J Immunol* 2003, 171:2402-2412.
84. Bonetti B, Valdo P, Ossi G, De Toni L, Masotto B, Marconi S, Rizzuto N, Nardelli E, Moretto G: T-cell cytotoxicity of human Schwann cells: TNFalpha promotes fasL-mediated apoptosis and IFN gamma perforin-mediated lysis. *Glia* 2003, 43:141-148.
85. O'Connell J: Fas ligand and the fate of antitumour cytotoxic T lymphocytes. *Immunology* 2002, 105:263-266.
86. Mehmut M, Takeda K, Abe M, Ogata H, Hirose S, Okumura K, Fujime M: Fas ligand and TNF-related apoptosis-inducing ligand induction on infiltrating lymphocytes in bladder carcinoma by bacillus Calmette-Guerin treatment. *Urol Int* 2005, 75:80-87.
87. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S: Lethal effect of the anti-Fas antibody in mice. *Nature* 1993, 364:806-809.
88. Schilling PJ, Murray JL, Markowitz AB: Novel tumor necrosis factor toxic effects. Pulmonary hemorrhage and severe hepatic dysfunction. *Cancer* 1992, 69:256-260.
89. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR, Yagita H: Nature's TRAIL--on a path to cancer immunotherapy. *Immunity* 2003, 18:1-6.
90. Almasan A, Ashkenazi A: Apo2L/TRAIL: apoptosis signaling, biology, and potential for cancer therapy. *Cytokine Growth Factor Rev* 2003, 14:337-348.
91. Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, et al.: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 1999, 5:157-163.
92. Wen J, Ramadevi N, Nguyen D, Perkins C, Worthington E, Bhalla K: Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. *Blood* 2000, 96:3900-3906.
93. Kim K, Fisher MJ, Xu SQ, el-Deiry WS: Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Cancer Res* 2000, 6:335-346.
94. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S: Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. *Cancer Res* 1999, 59:734-741.

95. Mariani SM, Krammer PH: Differential regulation of TRAIL and CD95 ligand in transformed cells of the T and B lymphocyte lineage. *Eur J Immunol* 1998, 28:973-982.
96. Wajant H, Moosmayer D, Wuest T, Bartke T, Gerlach E, Schonherr U, Peters N, Scheurich P, Pfizenmaier K: Differential activation of TRAIL-R1 and -2 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by a soluble TRAIL derivative. *Oncogene* 2001, 20:4101-4106.
97. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, Ashkenazi A, de Vos AM: Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell* 1999, 4:563-571.
98. Mongkolsapaya J, Grimes JM, Chen N, Xu XN, Stuart DI, Jones EY, Screaton GR: Structure of the TRAIL-DR5 complex reveals mechanisms conferring specificity in apoptotic initiation. *Nat Struct Biol* 1999, 6:1048-1053.
99. Rieger J, Ohgaki H, Kleihues P, Weller M: Human astrocytic brain tumors express AP02L/TRAIL. *Acta Neuropathol (Berl)* 1999, 97:1-4.
100. Zhang XD, Franco AV, Nguyen T, Gray CP, Hersey P: Differential localization and regulation of death and decoy receptors for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. *J Immunol* 2000, 164:3961-3970.
101. Bodmer JL, Meier P, Tschopp J, Schneider P: Cysteine 230 is essential for the structure and activity of the cytotoxic ligand TRAIL. *J Biol Chem* 2000, 275:20632-20637.
102. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM: An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 1997, 277:815-818.
103. Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J, Dixit VM: The receptor for the cytotoxic ligand TRAIL. *Science* 1997, 276:111-113.
104. Schneider P, Bodmer JL, Thome M, Hofmann K, Holler N, Tschopp J: Characterization of two receptors for TRAIL. *FEBS Lett* 1997, 416:329-334.
105. Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI: TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. *Curr Biol* 1997, 7:693-696.
106. Degli-Esposti MA, Smolak PJ, Walczak H, Waugh J, Huang CP, DuBose RF, Goodwin RG, Smith CA: Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med* 1997, 186:1165-1170.
107. Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA, Goodwin RG: The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* 1997, 7:813-820.
108. Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P, et al.: A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol* 1997, 7:1003-1006.

109. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, et al.: Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* 1998, 273:14363-14367.
110. Truneh A, Sharma S, Silverman C, Khandekar S, Reddy MP, Deen KC, McLaughlin MM, Srinivasula SM, Livi GP, Marshall LA, et al.: Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. *J Biol Chem* 2000, 275:23319-23325.
111. MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES: Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem* 1997, 272:25417-25420.
112. Burns TF, Bernhard EJ, El-Deiry WS: Tissue specific expression of p53 target genes suggests a key role for KILLER/DR5 in p53-dependent apoptosis in vivo. *Oncogene* 2001, 20:4601-4612.
113. Takimoto R, El-Deiry WS: Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. *Oncogene* 2000, 19:1735-1743.
114. Nimmanapalli R, Perkins CL, Orlando M, O'Bryan E, Nguyen D, Bhalla KN: Pretreatment with paclitaxel enhances apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of prostate cancer cells by inducing death receptors 4 and 5 protein levels. *Cancer Res* 2001, 61:759-763.
115. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, et al.: Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 1997, 277:818-821.
116. Pan G, Ni J, Yu G, Wei YF, Dixit VM: TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* 1998, 424:41-45.
117. Leverkus M, Neumann M, Mengling T, Rauch CT, Brocker EB, Krammer PH, Walczak H: Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. *Cancer Res* 2000, 60:553-559.
118. Hao C, Beguinot F, Condorelli G, Trencia A, Van Meir EG, Yong VW, Parney IF, Roa WH, Petruk KC: Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apotosis in human malignant glioma cells. *Cancer Res* 2001, 61:1162-1170.
119. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, et al.: Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* 1997, 89:309-319.
120. Pritzker LB, Scatena M, Giachelli CM: The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Biol Cell* 2004, 15:2834-2841.
121. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME: Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* 1995, 14:5579-5588.

122. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM: FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995, 81:505-512.
123. Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH, Wallach D: A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J Biol Chem* 1995, 270:7795-7798.
124. Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, Blenis J, Tschopp J: TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* 2000, 2:241-243.
125. Kuang AA, Diehl GE, Zhang J, Winoto A: FADD is required for DR4- and DR5-mediated apoptosis: lack of trail-induced apoptosis in FADD-deficient mouse embryonic fibroblasts. *J Biol Chem* 2000, 275:25065-25068.
126. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, et al.: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* 1996, 85:817-827.
127. Samali A, Cai J, Zhivotovsky B, Jones DP, Orrenius S: Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells. *Embo J* 1999, 18:2040-2048.
128. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, et al.: Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995, 376:37-43.
129. Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G, et al.: In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci U S A* 1996, 93:7464-7469.
130. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Litwack G, Alnemri ES: Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proc Natl Acad Sci U S A* 1996, 93:14486-14491.
131. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME: Two CD95 (APO-1/Fas) signaling pathways. *Embo J* 1998, 17:1675-1687.
132. Ekedahl J, Joseph B, Grigoriev MY, Muller M, Magnusson C, Lewensohn R, Zhivotovsky B: Expression of inhibitor of apoptosis proteins in small- and non-small-cell lung carcinoma cells. *Exp Cell Res* 2002, 279:277-290.
133. Wagenknecht B, Glaser T, Naumann U, Kugler S, Isenmann S, Bahr M, Korneluk R, Liston P, Weller M: Expression and biological activity of X-linked inhibitor of apoptosis (XIAP) in human malignant glioma. *Cell Death Differ* 1999, 6:370-376.
134. Deng Y, Lin Y, Wu X: TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/DIABLO. *Genes Dev* 2002, 16:33-45.
135. Li H, Zhu H, Xu CJ, Yuan J: Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998, 94:491-501.

136. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ: BID: a novel BH3 domain-only death agonist. *Genes Dev* 1996, 10:2859-2869.
137. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ: Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem* 1999, 274:1156-1163.
138. Madesh M, Antonsson B, Srinivasula SM, Alnemri ES, Hajnoczky G: Rapid kinetics of tBid-induced cytochrome c and Smac/DIABLO release and mitochondrial depolarization. *J Biol Chem* 2002, 277:5651-5659.
139. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH: Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ* 2000, 7:1166-1173.
140. Schendel SL, Azimov R, Pawlowski K, Godzik A, Kagan BL, Reed JC: Ion channel activity of the BH3 only Bcl-2 family member, BID. *J Biol Chem* 1999, 274:21932-21936.
141. Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ: Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 1999, 400:886-891.
142. Kroemer G, Reed JC: Mitochondrial control of cell death. *Nat Med* 2000, 6:513-519.
143. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D, Ashkenazi A: Tumor-cell resistance to death receptor--induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nat Med* 2002, 8:274-281.
144. Hinz S, Trauzold A, Boenicke L, Sandberg C, Beckmann S, Bayer E, Walczak H, Kalthoff H, Ungefroren H: Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. *Oncogene* 2000, 19:5477-5486.
145. Gazzaniga P, Gradilone A, Vercillo R, Gandini O, Silvestri I, Napolitano M, Albonici L, Vincenzoni A, Gallucci M, Frati L, et al.: Bcl-2/bax mRNA expression ratio as prognostic factor in low-grade urinary bladder cancer. *Int J Cancer* 1996, 69:100-104.
146. Pollack A, Wu CS, Czerniak B, Zagars GK, Benedict WF, McDonnell TJ: Abnormal bcl-2 and pRb expression are independent correlates of radiation response in muscle-invasive bladder cancer. *Clin Cancer Res* 1997, 3:1823-1829.
147. Ye D, Li H, Qian S, Sun Y, Zheng J, Ma Y: bcl-2/bax expression and p53 gene status in human bladder cancer: relationship to early recurrence with intravesical chemotherapy after resection. *J Urol* 1998, 160:2025-2028; discussion 2029.
148. Miyake H, Hanada N, Nakamura H, Kagawa S, Fujiwara T, Hara I, Eto H, Gohji K, Arakawa S, Kamidono S, et al.: Overexpression of Bcl-2 in bladder cancer cells inhibits apoptosis induced by cisplatin and adenoviral-mediated p53 gene transfer. *Oncogene* 1998, 16:933-943.

149. Kong G, Shin KY, Oh YH, Lee JJ, Park HY, Woo YN, Lee JD: Bcl-2 and p53 expressions in invasive bladder cancers. *Acta Oncol* 1998, 37:715-720.
150. Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM: The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* 1985, 229:1390-1393.
151. McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP, Korsmeyer SJ: bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* 1989, 57:79-88.
152. Gross A, McDonnell JM, Korsmeyer SJ: BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999, 13:1899-1911.
153. Wang X: The expanding role of mitochondria in apoptosis. *Genes Dev* 2001, 15:2922-2933.
154. Horvitz HR: Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer Res* 1999, 59:1701s-1706s.
155. Cory S, Adams JM: The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002, 2:647-656.
156. Thomas WD, Zhang XD, Franco AV, Nguyen T, Hersey P: TNF-related apoptosis-inducing ligand-induced apoptosis of melanoma is associated with changes in mitochondrial membrane potential and perinuclear clustering of mitochondria. *J Immunol* 2000, 165:5612-5620.
157. Sun SY, Yue P, Zhou JY, Wang Y, Choi Kim HR, Lotan R, Wu GS: Overexpression of BCL2 blocks TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in human lung cancer cells. *Biochem Biophys Res Commun* 2001, 280:788-797.
158. Gallo O, Boddi V, Calzolari A, Simonetti L, Trovati M, Bianchi S: bcl-2 protein expression correlates with recurrence and survival in early stage head and neck cancer treated by radiotherapy. *Clin Cancer Res* 1996, 2:261-267.
159. King ED, Matteson J, Jacobs SC, Kyprianou N: Incidence of apoptosis, cell proliferation and bcl-2 expression in transitional cell carcinoma of the bladder: association with tumor progression. *J Urol* 1996, 155:316-320.
160. Bilim V, Tomita Y, Kawasaki T, Katagiri A, Imai T, Takeda M, Takahashi K: Prognostic value of Bcl-2 and p53 expression in urinary tract transitional cell cancer. *J Natl Cancer Inst* 1996, 88:686-688.
161. Sandur SK, Ichikawa H, Sethi G, Ahn KS, Aggarwal BB: Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and IkappaBalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. *J Biol Chem* 2006, 281:17023-17033.
162. Rokhlin OW, Glover RA, Taghiyev AF, Guseva NV, Seftor RE, Shyshynova I, Gudkov AV, Cohen MB: Bisindolylmaleimide IX facilitates tumor necrosis factor receptor family-mediated cell death and acts as an inhibitor of transcription. *J Biol Chem* 2002, 277:33213-33219.
163. Shishodia S, Gutierrez AM, Lotan R, Aggarwal BB: N-(4-hydroxyphenyl)retinamide inhibits invasion, suppresses osteoclastogenesis, and potentiates apoptosis through down-regulation of I(kappa)B(alpha)

kinase and nuclear factor-kappaB-regulated gene products. *Cancer Res* 2005, 65:9555-9565.

164. Miyake H, Gleave M, Kamidono S, Hara I: Overexpression of clusterin in transitional cell carcinoma of the bladder is related to disease progression and recurrence. *Urology* 2002, 59:150-154.
165. Blaschuk O, Burdzy K, Fritz IB: Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *J Biol Chem* 1983, 258:7714-7720.
166. Jenne DE, Tschopp J: Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc Natl Acad Sci U S A* 1989, 86:7123-7127.
167. Kirszbaum L, Sharpe JA, Murphy B, d'Apice AJ, Classon B, Hudson P, Walker ID: Molecular cloning and characterization of the novel, human complement-associated protein, SP-40,40: a link between the complement and reproductive systems. *Embo J* 1989, 8:711-718.
168. de Silva HV, Harmony JA, Stuart WD, Gil CM, Robbins J: Apolipoprotein J: structure and tissue distribution. *Biochemistry* 1990, 29:5380-5389.
169. Poon S, Easterbrook-Smith SB, Rybchyn MS, Carver JA, Wilson MR: Clusterin is an ATP-independent chaperone with very broad substrate specificity that stabilizes stressed proteins in a folding-competent state. *Biochemistry* 2000, 39:15953-15960.
170. Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, Solary E: HSP27 inhibits cytochrome c-dependent activation of procaspase-9. *Faseb J* 1999, 13:2061-2070.
171. Bruey JM, Ducasse C, Bonniaud P, Ravagnan L, Susin SA, Diaz-Latoud C, Gurbuxani S, Arrigo AP, Kroemer G, Solary E, et al.: Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat Cell Biol* 2000, 2:645-652.
172. Sintich SM, Steinberg J, Kozlowski JM, Lee C, Pruden S, Sayeed S, Sensibar JA: Cytotoxic sensitivity to tumor necrosis factor-alpha in PC3 and LNCaP prostatic cancer cells is regulated by extracellular levels of SGP-2 (clusterin). *Prostate* 1999, 39:87-93.
173. Cervellera M, Raschella G, Santilli G, Tanno B, Ventura A, Mancini C, Sevignani C, Calabretta B, Sala A: Direct transactivation of the anti-apoptotic gene apolipoprotein J (clusterin) by B-MYB. *J Biol Chem* 2000, 275:21055-21060.
174. Wilson MR, Easterbrook-Smith SB: Clusterin is a secreted mammalian chaperone. *Trends Biochem Sci* 2000, 25:95-98.
175. Han BH, DeMattos RB, Dugan LL, Kim-Han JS, Brendza RP, Fryer JD, Kierson M, Cirrito J, Quick K, Harmony JA, et al.: Clusterin contributes to caspase-3-independent brain injury following neonatal hypoxia-ischemia. *Nat Med* 2001, 7:338-343.
176. Humphreys D, Hochgrebe TT, Easterbrook-Smith SB, Tenniswood MP, Wilson MR: Effects of clusterin overexpression on TNFalpha- and TGFbeta-mediated death of L929 cells. *Biochemistry* 1997, 36:15233-15243.

177. Lakins J, Bennett SA, Chen JH, Arnold JM, Morrissey C, Wong P, O'Sullivan J, Tenniswood M: Clusterin biogenesis is altered during apoptosis in the regressing rat ventral prostate. *J Biol Chem* 1998, 273:27887-27895.
178. Akakura K, Bruchovsky N, Rennie PS, Coldman AJ, Goldenberg SL, Tenniswood M, Fox K: Effects of intermittent androgen suppression on the stem cell composition and the expression of the TRPM-2 (clusterin) gene in the Shionogi carcinoma. *J Steroid Biochem Mol Biol* 1996, 59:501-511.
179. Redondo M, Villar E, Torres-Munoz J, Tellez T, Morell M, Petito CK: Overexpression of clusterin in human breast carcinoma. *Am J Pathol* 2000, 157:393-399.
180. Zhang H, Kim JK, Edwards CA, Xu Z, Taichman R, Wang CY: Clusterin inhibits apoptosis by interacting with activated Bax. *Nat Cell Biol* 2005, 7:909-915.
181. Reddy KB, Karode MC, Harmony AK, Howe PH: Interaction of transforming growth factor beta receptors with apolipoprotein J/clusterin. *Biochemistry* 1996, 35:309-314.
182. Trougakos IP, Gonos ES: Clusterin/apolipoprotein J in human aging and cancer. *Int J Biochem Cell Biol* 2002, 34:1430-1448.
183. Kadomatsu K, Anzano MA, Slayter MV, Winokur TS, Smith JM, Sporn MB: Expression of sulfated glycoprotein 2 is associated with carcinogenesis induced by N-nitroso-N-methylurea in rat prostate and seminal vesicle. *Cancer Res* 1993, 53:1480-1483.
184. Parczyk K, Pilarsky C, Rachel U, Koch-Brandt C: Gp80 (clusterin; TRPM-2) mRNA level is enhanced in human renal clear cell carcinomas. *J Cancer Res Clin Oncol* 1994, 120:186-188.
185. Steinberg J, Oyasu R, Lang S, Sintich S, Rademaker A, Lee C, Kozlowski JM, Sensibar JA: Intracellular levels of SGP-2 (Clusterin) correlate with tumor grade in prostate cancer. *Clin Cancer Res* 1997, 3:1707-1711.
186. Jaattela M: Escaping cell death: survival proteins in cancer. *Exp Cell Res* 1999, 248:30-43.
187. Allen RG, Tresini M, Keogh BP, Doggett DL, Cristofalo VJ: Differences in electron transport potential, antioxidant defenses, and oxidant generation in young and senescent fetal lung fibroblasts (WI-38). *J Cell Physiol* 1999, 180:114-122.
188. Miyake H, Hara I, Kamidono S, Gleave ME: Synergistic chemsensitization and inhibition of tumor growth and metastasis by the antisense oligodeoxynucleotide targeting clusterin gene in a human bladder cancer model. *Clin Cancer Res* 2001, 7:4245-4252.
189. Milligan JF, Matteucci MD, Martin JC: Current concepts in antisense drug design. *J Med Chem* 1993, 36:1923-1937.
190. Helene C, Toulme JJ: Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim Biophys Acta* 1990, 1049:99-125.
191. Weintraub HM: Antisense RNA and DNA. *Sci Am* 1990, 262:40-46.
192. Eder PS, DeVine RJ, Dagle JM, Walder JA: Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3' exonuclease in plasma. *Antisense Res Dev* 1991, 1:141-151.

193. Miller PS, Ts'o PO: A new approach to chemotherapy based on molecular biology and nucleic acid chemistry: Matagen (masking tape for gene expression). *Anticancer Drug Des* 1987, 2:117-128.
194. Miller P: Antisense oligonucleotide methylphosphonates. In Anitsense RNA and DNA, ed. JAH Murray, . *New York:Wiley-Liss* 1992:241-253.
195. Eckstein F: Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev* 2000, 10:117-121.
196. Geary RS, Yu RZ, Levin AA: Pharmacokinetics of phosphorothioate antisense oligodeoxynucleotides. *Curr Opin Investig Drugs* 2001, 2:562-573.
197. Akhtar S, Basu S, Wickstrom E, Juliano RL: Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). *Nucleic Acids Res* 1991, 19:5551-5559.
198. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M: Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* 1987, 84:7413-7417.
199. Beltinger C, Saragovi HU, Smith RM, LeSauteur L, Shah N, DeDionisio L, Christensen L, Raible A, Jarett L, Gewirtz AM: Binding, uptake, and intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides. *J Clin Invest* 1995, 95:1814-1823.
200. Hawley P, Gibson I: Interaction of oligodeoxynucleotides with mammalian cells. *Antisense Nucleic Acid Drug Dev* 1996, 6:185-195.
201. Brown DA, Kang SH, Gryaznov SM, DeDionisio L, Heidenreich O, Sullivan S, Xu X, Nerenberg MI: Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. *J Biol Chem* 1994, 269:26801-26805.
202. Stein CA: Two problems in antisense biotechnology: in vitro delivery and the design of antisense experiments. *Biochim Biophys Acta* 1999, 1489:45-52.
203. Guvakova MA, Yakubov LA, Vlodavsky I, Tonkinson JL, Stein CA: Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. *J Biol Chem* 1995, 270:2620-2627.
204. Sarmiento UM, Perez JR, Becker JM, Narayanan R: In vivo toxicological effects of rel A antisense phosphorothioates in CD-1 mice. *Antisense Res Dev* 1994, 4:99-107.
205. Agrawal S, Rustagi PK, Shaw DR: Novel enzymatic and immunological responses to oligonucleotides. *Toxicol Lett* 1995, 82-83:431-434.
206. Monia BP: First- and second-generation antisense inhibitors targeted to human c-raf kinase: in vitro and in vivo studies. *Anticancer Drug Des* 1997, 12:327-339.
207. Chiang MY, Chan H, Zounes MA, Freier SM, Lima WF, Bennett CF: Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J Biol Chem* 1991, 266:18162-18171.
208. Cotter FE: Antisense therapy of hematologic malignancies. *Semin Hematol* 1999, 36:9-14.

209. Miayake H, Tolcher A, Gleave ME: Chemosensitization and delayed androgen-independent recurrence of prostate cancer with the use of antisense Bcl-2 oligodeoxynucleotides. *J Natl Cancer Inst* 2000, 92:34-41.
210. Leech SH, Olie RA, Gautschi O, Simoes-Wust AP, Tschopp S, Haner R, Hall J, Stahel RA, Zangemeister-Wittke U: Induction of apoptosis in lung-cancer cells following bcl-xL anti-sense treatment. *Int J Cancer* 2000, 86:570-576.
211. Bilim V, Kasahara T, Noboru H, Takahashi K, Tomita Y: Caspase involved synergistic cytotoxicity of bcl-2 antisense oligonucleotides and adriamycin on transitional cell cancer cells. *Cancer Lett* 2000, 155:191-198.
212. Wallace TL, Bazemore SA, Kornbrust DJ, Cossum PA: Single-dose hemodynamic toxicity and pharmacokinetics of a partial phosphorothioate anti-HIV oligonucleotide (AR177) after intravenous infusion to cynomolgus monkeys. *J Pharmacol Exp Ther* 1996, 278:1306-1312.
213. Henry SP, Novotny W, Leeds J, Auletta C, Kornbrust DJ: Inhibition of coagulation by a phosphorothioate oligonucleotide. *Antisense Nucleic Acid Drug Dev* 1997, 7:503-510.
214. Flaherty KT, Stevenson JP, O'Dwyer PJ: Antisense therapeutics: lessons from early clinical trials. *Curr Opin Oncol* 2001, 13:499-505.
215. Kilani RT, Tamimi Y, Karmali S, Mackey J, Hanel EG, Wong KK, Moore RB: Selective cytotoxicity of gemcitabine in bladder cancer cell lines. *Anticancer Drugs* 2002, 13:557-566.
216. Griffith TS, Lynch DH: TRAIL: a molecule with multiple receptors and control mechanisms. *Curr Opin Immunol* 1998, 10:559-563.
217. Deveraux QL, Reed JC: IAP family proteins--suppressors of apoptosis. *Genes Dev* 1999, 13:239-252.
218. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, et al.: Inhibition of death receptor signals by cellular FLIP. *Nature* 1997, 388:190-195.
219. Song JH, Tse MC, Bellail A, Phuphanich S, Khuri F, Kneteman NM, Hao C: Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. *Cancer Res* 2007, 67:6946-6955.
220. Hao C, Song JH, Hsi B, Lewis J, Song DK, Petruk KC, Tyrrell DL, Kneteman NM: TRAIL inhibits tumor growth but is nontoxic to human hepatocytes in chimeric mice. *Cancer Res* 2004, 64:8502-8506.
221. Lawrence D, Shahrokh Z, Marsters S, Achilles K, Shih D, Mounho B, Hillan K, Totpal K, DeForge L, Schow P, et al.: Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* 2001, 7:383-385.
222. Kelley SK, Harris LA, Xie D, Deforge L, Totpal K, Bussiere J, Fox JA: Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther* 2001, 299:31-38.
223. Trougakos IP, So A, Jansen B, Gleave ME, Gonos ES: Silencing expression of the clusterin/apolipoprotein j gene in human cancer cells using small interfering RNA induces spontaneous apoptosis, reduced growth ability, and

cell sensitization to genotoxic and oxidative stress. *Cancer Res* 2004, 64:1834-1842.